STIC-ILL

RBI.L2

364226

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
T : STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 49-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

4399532

3282185

0023-6837/88/5804-0365$02.00/0
LABORATORY INVESTIGATION

Vol. 58, No. 4, p. 365, 1988
Printed in U.S.A.

Biology of Disease

Acute Inflammation and Microthrombosis Induced by Endotoxin, Interleukin-1, and Tumor Necrosis Factor and their Implication in Gram-Negative Infection

MYRON I. CYBULSKY, M. K. WILLIAM CHAN, AND HENRY Z. MOVAT

Departments of Pathology and of Immunology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Introduction 365
Inflammation Induced by *E. coli* and Endotoxin 365
Morphologic observations 365
Quantitative studies 366
Mediation of Endotoxin-Induced Inflammation 367
Leukocyte chemoattractants 367
Cytokines: mediators of the acute phase response 367
Interleukin 1: mediator of endotoxin-induced neutrophil emigration 368
Endotoxin and cytokine-induced endothelial cell adhesiveness for leukocytes 369
Endotoxin-Induced Microvascular Injury and Thrombosis 370
Inflammation and Host Defense in Gram-Negative Infection 373

INTRODUCTION

Acute inflammation constitutes the body's principal mode of defense against infection and other harmful agents, and neutrophils are the primary effector cells in this process. When inflammation occurs in response to infection with pathogenic microorganisms, the damage that is often observed locally is a sacrifice aimed to prevent the spread of infectious agents throughout the body. Gram-negative microorganisms elicit a brisk inflammatory reaction which is largely induced by one of their cell wall constituents, endotoxin. The infiltrating neutrophils phagocytose and kill the bacteria. The inflammatory reaction is often associated with severe local microvascular injury and abscess formation. Besides eliciting inflammation, endotoxin can predispose the local microvasculature to thrombosis upon subsequent systemic endotoxemia or complement activation, as demonstrated by the local Shwartzman reaction. Both the inflammatory and the thrombotic phenomena induced by endotoxin are mediated by the local generation of cytokines.

In addition to local effects, endotoxin shed by Gram-negative bacteria can access the circulation resulting in profound systemic effects. Endotoxin has been suggested as the principal causative agent of Gram-negative septic shock and disseminated intravascular coagulation, which are associated with a high mortality. It is also capable of eliciting fever, various components of the acute phase reaction, and a prolonged and profound neutropenia.

In this review, we examine the inflammatory and thrombotic reactions elicited by a Gram-negative bacterium, *Escherichia coli* and by endotoxin, followed by an analysis of the *in vivo* and *in vitro* observations which implicate cytokines as the mediators of these phenomena. We then examine the type and the mechanisms of the resulting microvascular injury and deal briefly with the significance of inflammation in defense against Gram-negative microorganisms.

INFLAMMATION INDUCED BY *E. coli* AND ENDOTOXIN

MORPHOLOGIC OBSERVATIONS

By counting the number of neutrophils in the lymph draining an inflammatory lesion and examining the tissue histologically, injection of *E. coli* was found to elicit a very intense inflammatory reaction, resulting in abscess formation in 24-hour lesions in sheep (64). Similar observations, including the abscess formation, were made subsequently in rabbits, in which ultrastructurally phagocytosed bacteria were demonstrable in neutrophils, often undergoing lysis (69). More recently, the morphology of the inflammatory lesions induced by killed *E. coli* was examined again and the findings correlated with other parameters (for details *vide infra* under "Inflammation and Host Defense in Gram-Negative Infection") (27). When 20 sites were injected simultaneously with *E. coli* (6×10^8/site), a marked neutropenia developed and

very few neutrophils were detectable at the injected site, and many extracellular bacteria were detectable histologically. A reinjection of the same large number of bacteria after recovery from the neutropenia (during the neutrophilic phase), resulted in a marked infiltration of the dermis by neutrophils, with very few bacteria, mostly within the phagocytes.

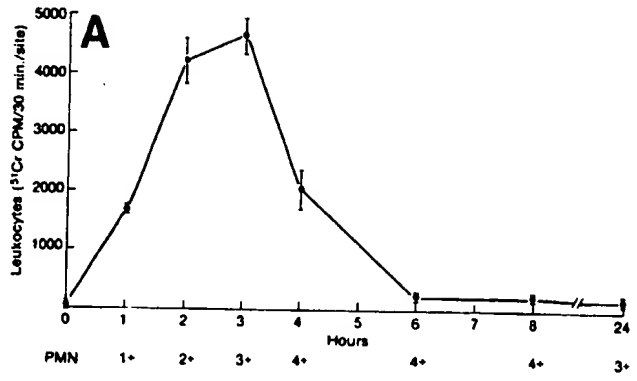
The injection of killed *E. coli* or large doses of endotoxin is followed by severe microvascular injury (*vide infra*, "Endotoxin-Induced Microvascular Injury and Thrombosis"). However, the injection of a large number of live *E. coli* (2×10^{10}/site) or smaller numbers in neutropenic rabbits is associated with necrosis of the dermis at the injection site (18).

Quantitative Studies

Having been able to quantitate increase in vasopermeability (115) and changes in blood flow in inflammation (49), it had become pertinent to quantitate the emigration of neutrophil leukocytes and their accumulation in the lesions (55, 56). The first studies on quantitation and kinetics of the acute inflammatory reaction were done with killed *E. coli*. Neutrophils were isolated from the blood of rabbits, radiolabeled and reinfused intravenously. These studies ascertained that the emigration of neutrophils was transient. The maximal rate of emigration was between 2 to 3 hours and after 6 to 8 hours, the rate of emigration was less than 10% of maximal (Fig. 1*A*). With live *E. coli* (18, 86) and leukocyte chemoattractants (22) neutrophil emigration followed similar kinetics. When blood mononuclear leukocytes were radiolabeled (61), the maximal rate of monocyte accumulation into lesions induced by killed *E. coli* coincided with neutrophils, however monocytes continued to accumulate at approximately 25% of the maximal rate for at least 24 hours (Fig. 1*B*). The absolute number of neutrophils which accumulate in *E. coli* lesions during the first 6 hours greatly exceeds the number of monocytes, as is evident in histologic sections of early lesions. Neutrophils comprise over 30% of the rabbit's circulating leukocytes, whereas monocytes constitute less than 5%. Thus, the delivery of circulating neutrophils to the inflammatory site exceeds monocytes by at least 6-fold. Emigrated neutrophils die relatively early or leave the inflammatory site via lymphatics (64). Monocytes continue to accumulate and undergo transformation into macrophages, thus becoming the predominant cell in the late stages of *E. coli* inflammation.

The skin of rabbits was ideal to quantitate events in an inflammatory reaction because various doses or time points under study could be injected in triplicate or quadruplicate, including positive and negative controls. However, *E. coli* or endotoxin as inflammatory stimuli were suitable also for the study of inflammation in the lung (29, 30) and pleura (53, 54). Moreover, by a further purification of the ^{51}Cr-labeled blood neutrophils, and a comparison of the radiolabeled cells in the blood and in a pleural exudate, the specificity and precision of the quantitative procedure was markedly improved (27). This enabled the expression of the results as the number of infiltrating neutrophils/lesion in relation to the number of circulating neutrophils. The latter was demonstrated to influence the delivery of neutrophils at the inflammatory site. When a large dose of *E. coli* was administered, a profound and prolonged neutropenia developed and neutrophil emigration into the *E. coli*-injected intradermal sites was markedly attenuated (27).

In addition to leukocyte emigration, the injection of *E. coli* induced a transient hyperemia, enhanced vasopermeability, and hemorrhage (69). *E. coli* and endotoxin



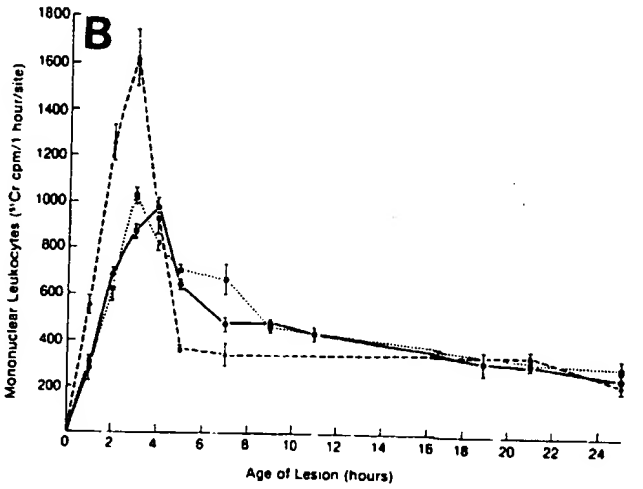


FIG. 1. Kinetics of leukocyte accumulation in rabbit skin. *A*, Rate of neutrophil leukocyte accumulation in inflammatory lesions induced by *E. coli*. Formalin-killed *E. coli* (5×10^8/site) were injected at varying times intradermally and autologous ^{51}Cr-labeled leukocytes were infused 30 minutes before sacrifice. Histologic examination ascertained that during peak emigration, over 90% of the infiltrating cell were neutrophil leukocytes (*PMNs*). Their number in hematoxylin and eosin-stained sections was scored semiquantitatively from 1 to 4+. This is one representative experiment of five. Points are means ± SEM of five replicate sites. Reproduced from Issekutz and Movat, Lab Invest 42:310, 1980. *B*, Mononuclear leukocyte accumulation in inflammatory lesions induced by *E. coli*. Killed *E. coli* (5×10^8/site) were injected intradermally at varying time intervals and ^{51}Cr-labeled mononuclear cells were injected intravenously 1 hour before sacrifice. The results of three experiments are shown. Points are mean ± SEM of triplicate sets. Saline-inoculated sites had 40 cpm, and this was subtracted from each point. By histology, the mononuclear cells were monocytes/macrophages. When ^{51}Cr-labeled lymphocytes were injected intravenously no accumulation was demonstrable. Reproduced from Issekutz *et al.*, Am J Pathol 103:47, 1981, Copyright by the American Association of Pathologists, Inc.

were the most frequent stimuli in inflammation associated with microhemorrhage (68) and microthrombosis (62, 87). Injected *E. coli* or endotoxin readily induce a hemorrhagic inflammatory reaction (69, 87), but this is seldom observed with staphylococcus-induced experimental inflammation (I. J. Cybulsky and H. Z. Movat, unpublished observations). Of all chemotaxins, only activated complement ($C5a_{des\ Arg}$) was capable of eliciting some hemorrhagic response (57, 58, 88). All the procedures mentioned above made use of radiolabeled cells and blood constituents for the quantitation of the inflammatory reaction.

Increase in vascular permeability and hemorrhage in inflammatory reactions induced by killed *E. coli* are neutrophil-dependent components of the reaction, since depletion of circulating neutrophils abrogates the development of vascular injury, quantitated with ^{125}I-albumin and ^{59}Fe-erythrocytes (70). This has been known for some time with respect to immune complex-induced inflammatory reactions associated with vascular injury (reviewed in Refs. 19, 81). The depletion of hemolytic complement and C5 markedly decreases neutrophil emigration, enhanced vasopermeability, and hemorrhage induced by the deposition of immune precipitates (24), however, it only partially inhibits the permeability change induced by *E. coli* and does not influence the neutrophil influx or hemorrhage (70). This suggested a mediating role for complement only in the immune complex-induced inflammatory reaction, and raised the question of a bacterium-derived substance in the mediation of *E. coli*-induced inflammation. Issekutz, Bhimji, and Bertolussi (52) demonstrated that killed *E. coli*-induced inflammation was diminished when the bacteria were treated with polymyxin B (which forms a complex with and inactivates endotoxin) or with anti-endotoxin antibody. Furthermore *E. coli* incubated in heat-inactivated plasma or buffer shed a substance into the supernatant, which induced neutrophil emigration when injected intradermally, but was not chemotactic *in vitro* (51). Polymyxin B or antibody to *O* or core glycolipid antigens diminished the potency of the supernatant (and of endotoxin) to induce neutrophil emigration. Issekutz and Bhimji (51) concluded that the material shed by the *E. coli* was endotoxin. Thus Gram-negative bacteria induce neutrophil emigration by releasing endotoxin which is not directly chemotactic or chemokinetic and at low doses exerts its effect *in vivo* independent of complement. During their growth phase *E. coli* secrete chemotactic formylated peptides (74) and potentially chemotactic lipids. Live *E. coli* induce also a neutrophil-independent tissue injury (18, 59), in which bacterial hemolysins play a role (59).

Attempts were also made to study the relationship between the movement of protein from the vasculature into the extracellular space and the clearance of this protein from the inflammatory site (induced by *E. coli* or other means). This was achieved by quantitating the increase in vascular permeability (^{131}I-albumin) and monitoring simultaneously the disappearance (clearance) of intradermally injected ^{125}I-albumin from inflammatory and control sites (47). It was observed that the removal rates of albumin injected into *E. coli*-induced inflammatory sites (10^2 to 10^6 *E. coli*/site) were not greater than those at sites injected with saline, despite 170 to 700% increases in vasopermeability observed in the inflammatory lesions. In fact, with high doses of *E. coli* (10^8/site) the mobilization of protein from the lesions was significantly reduced. In contrast with other inflammatory stimuli (bradykinin, heat injury) the clearance of extravascular protein from the lesions was enhanced over saline sites, implying a unique mechanism with *E. coli* (and endotoxin)-induced inflammatory edema (47). These observations may have relevance to the development of edema associated with Gram-negative septicemia and have led to studies on the role of lymphatic vessels in inflammatory edema (38, 63).

MEDIATION OF ENDOTOXIN-INDUCED INFLAMMATION

Gram-negative bacteria release (shed) endotoxins, which are lipopolysaccharide-protein complexes (40, 79, 120) and elicit a marked acute inflammatory reaction characterized by intense neutrophil emigration. The hypothesis that endotoxin-induced neutrophil emigration is a mediated process, is based on the knowledge that many of the *in vivo* biologic effects of endotoxin are mediated by host-derived mediators: leukocyte chemoattractants (C5a) and cytokines.

Leukocyte Chemoattractants

To date, substantial evidence has accumulated which suggest that leukocyte chemoattractants do not mediate endotoxin-induced neutrophil emigration. The depletion of complement with cobra venom factor had minimal effects on emigration induced by endotoxin (28) or *E. coli* (70), which indicates that C5a is not a relevant mediator. Picogram quantities of endotoxin are sufficient to elicit detectable neutrophil infiltration into the skin of rabbits and comparisons of molar potencies to leukocyte chemoattractants estimated that endotoxin was at least 1000-fold more potent (22, 26). Moreover, endotoxin did not induce tachyphylaxis to several leukocyte chemoattractants (20, 21, 26). The experiments of McComb, Cybulsky, and Movat (76) demonstrated that endotoxin induces neutrophil emigration by a different mechanism than leukocyte chemoattractants. The onset of neutrophil emigration following the injection of leukocyte chemoattractants was found to be more rapid and unlike endotoxin was not dependent on protein synthesis.

Cytokines: Mediators of the Acute Phase Response

The concept that cytokines are mediators of endotoxin-induced inflammation arose from observations made in conjunction with the acute phase response, which followed various forms of trauma and tissue injury associated with inflammation, but particularly the injection of endotoxin. The acute phase reaction includes fever, leukopenia followed by leukocytosis with neutrophilia, changes in certain heavy metals in the plasma, increase in acute phase proteins (e.g., C-reactive protein,

plasma proteinase inhibitors, transport proteins, fibrinogen) in plasma and their secretion in the liver (reviewed in Refs. 67, 81). Kampschmidt and co-workers described in the 60s and 70s a substance, "leukocytic endogenous mediator" which like the earlier describ d "endogenous pyrogen" and endotoxin, had the capacity to elicit the acute phase response (reviewed in Ref. 65). Both endogenous pyrogen and leukocytic endogenous mediator were shown to be leukocyte-derived, but the cell type was first in question, until monocytes/macrophages were demonstrated as the primary source of interleukin-1 (IL-1), endogenous pyrogen and lymphocyte-activating factor (2, 48, 121). However, more recently good evidence was again presented that neutrophils can synthesize IL-1 (122). Another important source of Il-1 with respect to inflammation is the endothelial cell (71, 92), and the smooth muscle cell (72). Lymphocyte-activating factor (in the thymocyte co-mitogenesis assay) was described in the 70s by immunologists and designated as IL-1 by a group of cellular immunologists (J Immunol 123:2928, 1979). Eventually endogenous pyrogen, leukocytic endogenous mediator and lymphocyte-activating factor were shown to be identical to IL-1, by comparing their biologic and biochemical properties (reviewed in Refs. 33, 34). Recently, tumor necrosis factor (TNF), a cytokine produced by monocytes-macrophages in response to endotoxin was found to possess many of the activities ascribed to IL-1 (6). In view of these observations, the roles of IL-1 and more recently of TNF in mediating neutrophil emigration induced by endotoxin were examined.

INTERLEUKIN 1: MEDIATOR OF ENDOTOXIN-INDUCED NEUTROPHIL EMIGRATION

Initial attention was focused on determining whether IL-1 could induce neutrophil emigration *in vivo*. Studies were carried out in which the activities in supernatants of cultured rabbit alevolar macrophages stimulated with opsonized zymosan or lipopolysaccharide, were assayed for neutrophil emigration-inducing activity and activity in the mouse thymocyte comitogenesis assay (25, 28). Fractions from a gel filtration column with low molecular weight showed activity in both assays, with peak activity in 14,000 to 16,000 molecular weight fractions, which corresponded to the MW of IL-1. High molecular weight fractions showed activity only in the neutrophil accumulation assay. Issekutz, Meygeri, and Issekutz (54) have reported similar activity in endotoxin-induced pleural exudates and supernatants of cultured pleural macrophages stimulated with endotoxin. Neutrophil emigration could also be induced with purified murine IL-1 (spirochete-stimulated macrophages, 5, 45), affinity-purified human monocyte-derived IL-1 (25, 26) and recombinant IL-1 preparations (28, 28a, 44, 96a). Observations with recombinant IL-1 are particularly important, since purified monocyte/macrophage-derived preparations may contain trace protein contaminants with biologic activities. Endotoxin contamination of IL-1 preparations has been ruled out by demonstrating stability of endotoxin but not of IL-1 to heating, and inhibition of endotoxin but not IL-1 activity with polymyxin B sulfate. Recently, TNF also has been found to induce neutrophil emigration (3, 28a, 31, 60, 85).

The analysis of the roles of IL-1 and TNF in endotoxin-induced neutrophil migration is based on (a) comparisons of potencies, (b) kinetic profiles and (c) examination of cross tachyphylaxis. The potencies of both IL-1α and IL-1β approached that of endotoxin (27, 28a) (Fig. 2) and are similar to synthetic lipid A (28a). TNF on the other hand was found to be less potent than the IL-1 species (28, 85) (Fig. 2) and of comparable molar potency to the leukocyte chemoattractants. In earlier studies, leukocyte chemoattractants were found to be at least 1000-fold less potent than endotoxin (21, 26). These experiments suggest that in order to elicit an equivalent magnitude of neutrophil emigration, each molecule of endotoxin would have to generate 1 to 10 molecules of IL-1, 1000 to 10,000 molecules of TNF, or over 1000 molecules of a leukocyte chemoattractant. The latter possibility is not likely at least with regards to $C5a_{des\,Arg}$ or leukotriene B_4 since relatively high concentrations of endotoxin are required for the activation of complement (51) and endotoxin is not a potent stimulator of leukotriene B_4 production (13). The relative amounts of IL-1and TNF production by different cell types remains to be determined, however if both are produced, their effects on neutrophil emigration appear to be synergistic (60, 85) (Fig. 2).

The kinetic profile of neutrophil emigration induced by IL-1, TNF or endotoxin demonstrated a very low rate of emigration in the first 30 minutes (28, 28a). This may represent the time required by the endothelial cells to synthesize proteins adhesive for leukocytes (*vide infra*). After 30 minutes, emigration into sites injected with the cytokines increased dramatically, but remained low for a further 30 minutes into sites injected with endotoxin. These observations suggest that endotoxin may induce the synthesis of a cytokine mediator *in situ*, a process requiring approximately 30 minutes, followed by a further 30 minutes for the synthesis of adhesive proteins by the endothelium. In contrast to the cytokines or endotoxin, leukocyte chemoattractants elicit neutrophil emi-

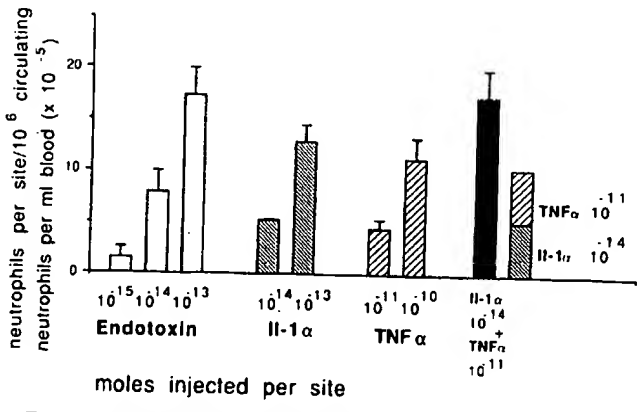


FIG. 2. Neutrophil emigration elicited with endotoxin, IL-1, TNFα or a mixture of the two cytokines, quantitated with ^{51}Cr-labeled neutrophils. The radiolabeled cells circulated for 2 hours after the intradermal injections and entered the inflammatory lesions during that time period. The dose response to two concentrations of IL-1α and TNFα are illustrated. When the lowest concentrations of the two cytokines were injected together, the neutrophil emigration was 69% greater than the additive response induced by each individual cytokine, implying a synergism between IL-1 and TNFα.

gration more rapidly, with significant emigration already detectable within the first 30 minutes after intradermal injections (76).

The term desensitization or tachyphylaxis at the inflammatory site was used in conjunction with reduced responsiveness to an inflammatory agent (20, 21). When dermal sites were reinjected with the same chemotaxin and the neutrophil emigration quantitated with ^{51}Cr-neutrophils after the reinjection (second injection), their number was decreased, compared with sites injected for the first time. Pertinent to this, was the observation that all inflammatory agents tested induced a transient emigration, which had ceased after the 4th hour (22). Thus, inflammatory lesions were initiated 8 hours before sacrifice and restimulated 6 hours later, followed immediately by the quantitation with the intravenously injected radiolabeled neutrophils. The tachyphylaxis was chemotaxin-specific, *i.e.*, sites initiated with *N*-formylmethionylleucylphenylalanine (FMLP) and restimulated with FMLP exhibited a reduced number of accumulated ^{51}Cr-neutrophils. In contrast, when FMLP-initiated sites were restimulated with platelet-activating factor, the same numbers of radiolabeled neutrophils accumulated as at sites injected for the first time with platelet-activating factor. The pathophysiologic role for tachyphylaxis may be to terminate inflammatory responses and to downregulate inflammation to chronically elevated (39) or physiologic (17) levels of cytokines. We utilized tachyphylaxis experiments to establish a potential role for cytokines generated *in situ* by endotoxin. Tachyphylaxis was noted at sites initiated and restimulated with endotoxin (21) and was observed also with natural IL-1 (26) and thereafter with recombinant IL-1 (27, 28a). Tachyphylaxis was dose-dependent; the higher the dose of the first stimulus, the fewer neutrophils accumulated after the reinjection.

Cross-tachyphylaxis was observed between IL-1α and IL-1β and between IL-1 and endotoxin (28a). When dermal sites were first stimulated with endotoxin and reinjected with IL-1, the response to IL-1 was reduced. Diminished neutrophil emigration was also observed when the order of endotoxin and IL-1 injections was reversed. This likely represents the desensitization of tissues to IL-1 generated in response to endotoxin, and implicates IL-1 as a potential mediator of endotoxin-induced neutrophil emigration. Cross-tachyphylaxis was not observed between TNF and endotoxin, suggesting that it is not a relevant mediator of neutrophil emigration.

In all these studies, the leukocytes infiltrating the dermis were almost exclusively neutrophils (Fig. 3).

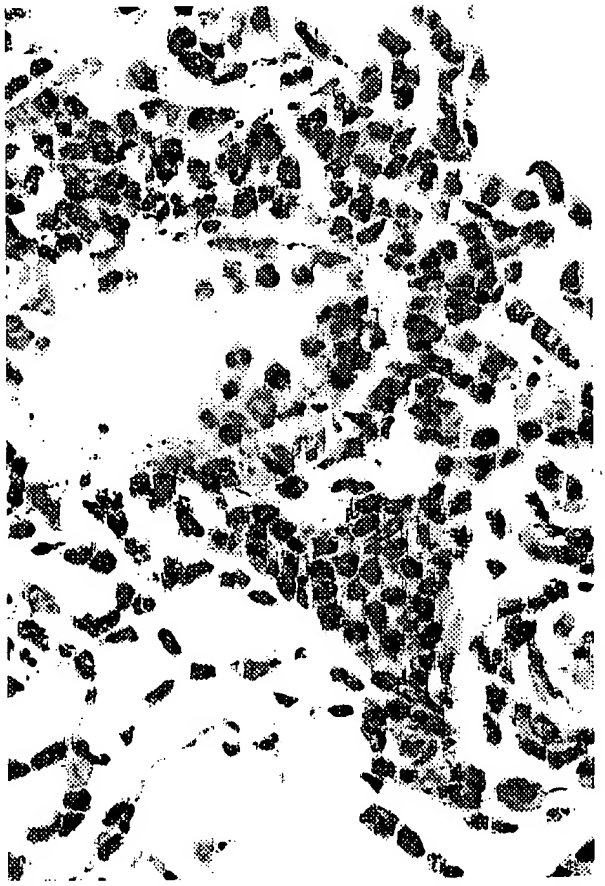


FIG. 3. Dermal inflammatory lesion (18 hours old) induced by the injection of 4 μg IL-1. Almost all the cells infiltrating the perivascular tissue are neutrophils. ×560.

ENDOTOXIN AND CYTOKINE-INDUCED ENDOTHELIAL CELL ADHESIVENESS FOR LEUKOCYTES

Leukocyte emigration into tissues through the walls of postcapillary venules and small veins can be divided into 3 steps: (a) adhesion of leukocytes to endothelial cells, (b) migration to intercellular junctions and diapedesis, (c) emigration through the endothelial basement membrane and vessel wall into extravascular tissues. An important regulatory step in emigration is the adhesion of leukocytes to endothelial cells, and is dependent both on endothelial and leukocyte mechanisms. Under certain physiologic conditions, leukocytes adhere weakly and transiently to the endothelium, which results in their rolling along venular walls: margination. Under pathologic conditions, particularly inflammation, margination is more pronounced until the leukocytes adhere strongly and eventually emigrate.

The advent of endothelial cell culture has led to *in vitro* studies of leukocyte-endothelial cell adhesion and elucidation of an endothelial cell-dependent adhesive mechanism. Both endotoxin and the cytokines IL-1, TNF and lymphotoxin induce endothelial cells to become adhesive for leukocytes by a mechanism dependent on protein synthesis (11, 37, 41, 103). Similarly, *in vivo* neutrophil emigration induced by endotoxin and IL-1 is dependent on protein synthesis, however this is not the case with leukocyte chemoattractants (76). A specific inducible endothelial cell surface protein designated by Gimbrone, Bevilacqua and colleagues as endothelial-leukocyte adhesion molecule 1 has been identified with two monoclonal antibodies: H4/8 (97) and H18/7 (10). It mediates, in part, neutrophil adherence to stimulated endothelial cells (10, 73). The induction of endothelial-leukocyte adhesion molecule 1 is transient, and endothelial cells become desensitized to restimulation with the

same cytokine or endotoxin, when the stimulus has been maintained in the culture medium (97, 98). *In vivo* endothelial-leukocyte adhesion molecule 1 has been identified in delayed type hypersensitivity reactions and in acute inflammatory conditions (23). The functional roles of other endothelial cell activation antigens (43) remain to be determined.

Leukocyte-dependent mechanisms for adhesion are predominantly mediated by a family of cell surface glycoproteins designated "LFA-1/Mac-1/p150,95" by Springer and co-workers (1,106) and CD11/CD18 by the Third International Workshop on Leukocyte Differentiation Antigens. These proteins are heterodimers with a distinct α-subunit (CD11a-c), noncovalently linked to a common β-subunit (CD18). Recurrent life-threatening bacterial infections developed by patients with hereditary deficiency of CD11/CD18 emphasizes its functional importance as reviewed by Anderson and Springer (1). Monoclonal antibodies to CD18 can block neutrophil adhesion to cytokine-stimulated endothelial cells (73, 99) and neutrophil emigration *in vivo* (1a).

ENDOTOXIN-INDUCED MICROVASCULAR INJURY AND THROMBOSIS

As described in the first section on quantitative studies, inflammatory reactions induced by *E. coli* or endotoxin are usually associated with severe injury, manifested by hemorrhage. It has been known for some time that endotoxin can elicit both local and systemic microvascular alterations, whose pathogenesis is not fully understood, and multiple mechanisms have been implicated (reviewed in Refs. 79, 80, 83).

Recent observations indicate that, like the neutrophil emigration, some of the microvascular alterations induced by endotoxin are mediated by IL-1 and TNF, and that neutrophils play a pivotal role in the development of the injury. As described in this review, neutrophil emigration can be induced by subnanogram quantities of endotoxin and both endotoxin and IL-1 are about 1000 times more potent in this respect than chemotaxins. For the elicitation of the classical local Shwartzman reaction, microgram quantities of endotoxin have to be injected locally ("preparative" injection), followed 18 to 24 hours later by an intravenous injection of endotoxin ("challenging" injection). The challenging injection of endotoxin could be substituted by a few procedures, but particularly by the intravascular activation of complement with immune precipitates, zymosan (82), or cobra venom factor (84). Rendering rabbits hypocomplementemic with cobra venom factor prevented the elicitation of a Shwartzman reaction when challenged with immune complexes or zymosan. However, when the intravenous challenge was endotoxin, the decomplementation induced only partial inhibition, implicating mainly complement-independent effects of the infused endotoxin (84). This substitution of endotoxin was more difficult with the preparative injection, until it was observed that with partially purified macrophage-derived IL-1 (presumably containing TNF) a Shwartzman-like reaction could be "prepared" (5, 84). However, when highly purified recombinant IL-1 was injected as a preparative dose, a thrombo-hemorrhagic Shwartzman-like reaction could only be elicited when recombinant TNF was injected simultaneously (84, 85). These lesions were quantitated with ^{59}Fe-erythrocytes (hemorrhage) and ^{111}In-platelets (thrombosis). Interestingly, sites prepared with endotoxin exhibited more thrombosis, and this was corroborated and extended morphologically. In addition to more numerous platelets, considerably more fibrin was seen in the lesions prepared with endotoxin than in those initiated with the cytokines. On the other hand, in the cytokine-induced lesions the hemorrhage was more intense.

These thrombo-hemorrhagic lesions which developed after the intravenous challenge, are superimposed on changes taking place locally after the preparative intradermal injections. In this respect, certain *in vitro* observations are important. These studies indicate that the endothelium plays an active role in coagulation, both in its promotion and inhibition (108). Both IL-1 and TNF can stimulate cultured endothelial cells to produce a tissue factor (thromboplastin)-like activity (8, 9, 93). By inhibiting fibrinolysis, IL-1 may play a role in the fate of a thrombus (12, 91). IL-1 and TNF added together to the endothelial monolayer were found to be additive with respect to induction of procoagulant activity, but *in vivo* with respect to the thrombo-hemorrhagic Shwartzman phenomenon they acted synergistically (84, 85). There is also *in vitro* evidence that endotoxin-stimulated endothelial cells synthesize IL-1 (71, 107). It is thus tempting to speculate that in addition to the inflammatory reaction induced by endotoxin via the cytokines at the injection sites, changes may be taking place at these sites which promote microthrombosis that develops after the intravenous challenge. It has been known for some time that intravenous injection, but particularly reinjection of endotoxin, leads to intravascular coagulation and consumption of clotting factors (80, 90). The reason why the microthrombi and hemorrhage were confined to the "prepared" site was the principal unanswered question, since the studies of Shwartzman (104). One decade ago innumerable intrinsic mediators were believed to be mediating events induced by endotoxin (79). Yet, none of these could fully account for its effects. Today we are still a long way from understanding the molecular events leading to a Shwartzman reaction, but the available evidence strongly suggests that IL-1 and TNF are the principal intrinsic mediators of this endotoxin-induced reaction.

Morphologic observations of the "prepared" sites immediately before intravenous challenge shed some light on the nature of these inflammatory lesions induced by endotoxin or IL-1 plus TNF, and thereby on the events which follow the intravenous challenge. Only when IL-1 and TNF were coinjected was the cellular infiltration comparable to that induced with endotoxin (31, 84, 85). Ultrastructurally, with endotoxin or IL-1 plus TNF, severe injury of venules and small veins was observed in conjunction with varying degrees of degenerative changes in neutrophils, both within and around the vessels. In these neutrophils granules had frequently fused with the cell membrane and were seen free in the lumen or abutting against endothelial cells. The latter exhibited focal or more extensive necrosis and frequently the basement membrane or remnants thereof was devoid of an endo-

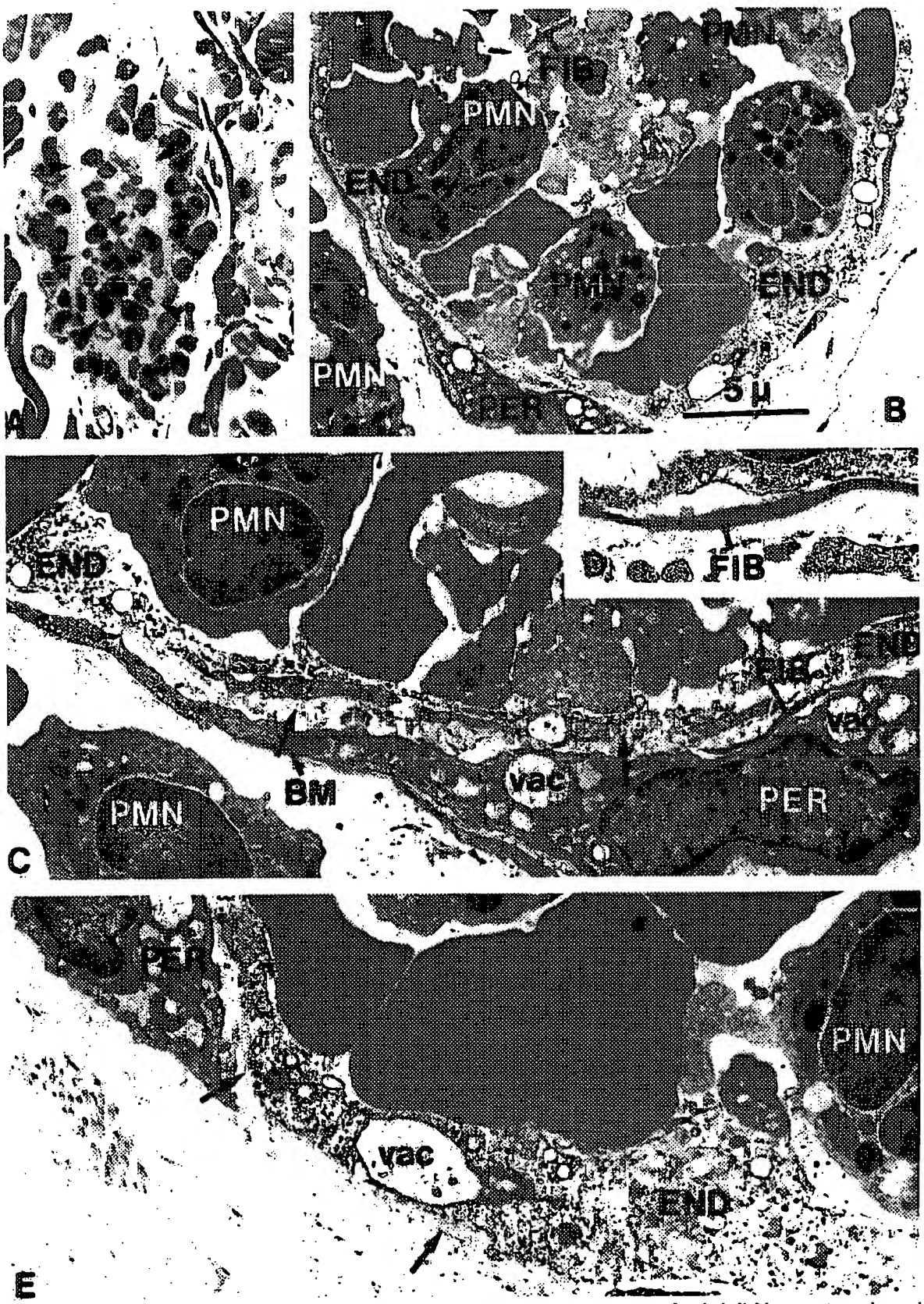


FIG. 4. Example of vessel wall injury. In Figure 4*A* (light microscopy) the endothelium is delineated by *arrowheads*. Neutrophils are present both in the lumen and perivascularly (inside and outside the *arrowheads*). About ½ the vessel is illustrated in the electron micrograph of *B*. Portions of the venular wall are reproduced in *C*, *D*, and *E*. The endothelial cells (*END*) are necrotic and the pericytes (*PER*) intact but vacuolated (*vac*). Some basement membrane (*BM*) surrounds pericytes, but the subendothelial basement membrane is fragmented or absent (*arrows*). Fibrin (*FIB*) is seen in the lumen of *B* and between endothelial cells and pericytes in *C* and *D* (*inset*). Figure 4*A*, ×560; *B*, × 4,125; *C*, ×12,000; *D*, ×33,900; *E*, ×12,000. Reproduced from Movat *et al.*, Am J Pathol 129:463, 1987, Copyright by the American Association of Pathologists, Inc.

thelial lining (Fig. 4). At times, the basement membrane was missing. Erythrocyte extravasation was associated with such changes (Fig. 5). This microvascular injury and the associated inflammatory hemorrhage gradually subsided. However, when a challenging intravenous injection of endotoxin, immune precipitates, zymosan, or cobra venom factor was administered at this time, microthrombi formed and occluded the vessels (Fig. 6). They consisted primarily of aggregated leukocytes, mostly neutrophils, but contained also aggregates of platelets and fibrin and were invariably accompanied by very extensive hemorrhage (85). The neutrophils in these thrombi also exhibited degenerative changes, but these were not seen in the monocytes and occasional lymphocytes. These events could be quantitated with radiolabeled cells and proteins. Early kinetic studies demonstrated that both thrombosis and hemorrhage developed rapidly and subsided 4 to 5 hours after the iv challenge (87).

The mediating role of IL-1 and TNF in the Shwartzman reaction is not fully understood as yet. Our studies on neutrophil emigration indicate that the cytokines, but particularly IL-1, are responsible for the neutrophil infiltration at the prepared site. The neutrophils are instrumental in the development of severe hemorrhage associated with the microvascular injury, because in neutropenic animals, a positive hemorrhagic reaction cannot be elicited (109) and because these cells showed signs of lysis and granule release. The only experimental model in which quantitatively and qualitatively, a similar vessel wall injury could be induced was that in which lysates of neutrophil lysosomes were injected intradermally (89).

There is no evidence in the literature that IL-1 can elicit hemorrhage, but TNF has been described to induce hemorrhagic necrosis of experimental tumors (101) and when massive doses (milligram quantities) were infused in rats, it elicited hemorrhage in several organs (114). Such quantities of TNF induce synthesis of IL-1 *in vitro* and *in vivo* (35), whereas stimulation with IL-1 induces production of IL-1, both *in vivo* and *in vitro* (36, 118). By itself even in very high doses, IL-1 was unable to "prepare" a site for the elicitation of a Shwartzman-like reaction; which was true also of TNF (84, 85). The term TNF is used interchangeably with TNFα and it is identical to cachectin, a substance implicated as the mediator of lethal endotoxin shock (6, 7, 114). By injecting merely microgram quantities, but both cytokines into rabbits, a synergism was demonstrated between IL-1 and TNF in

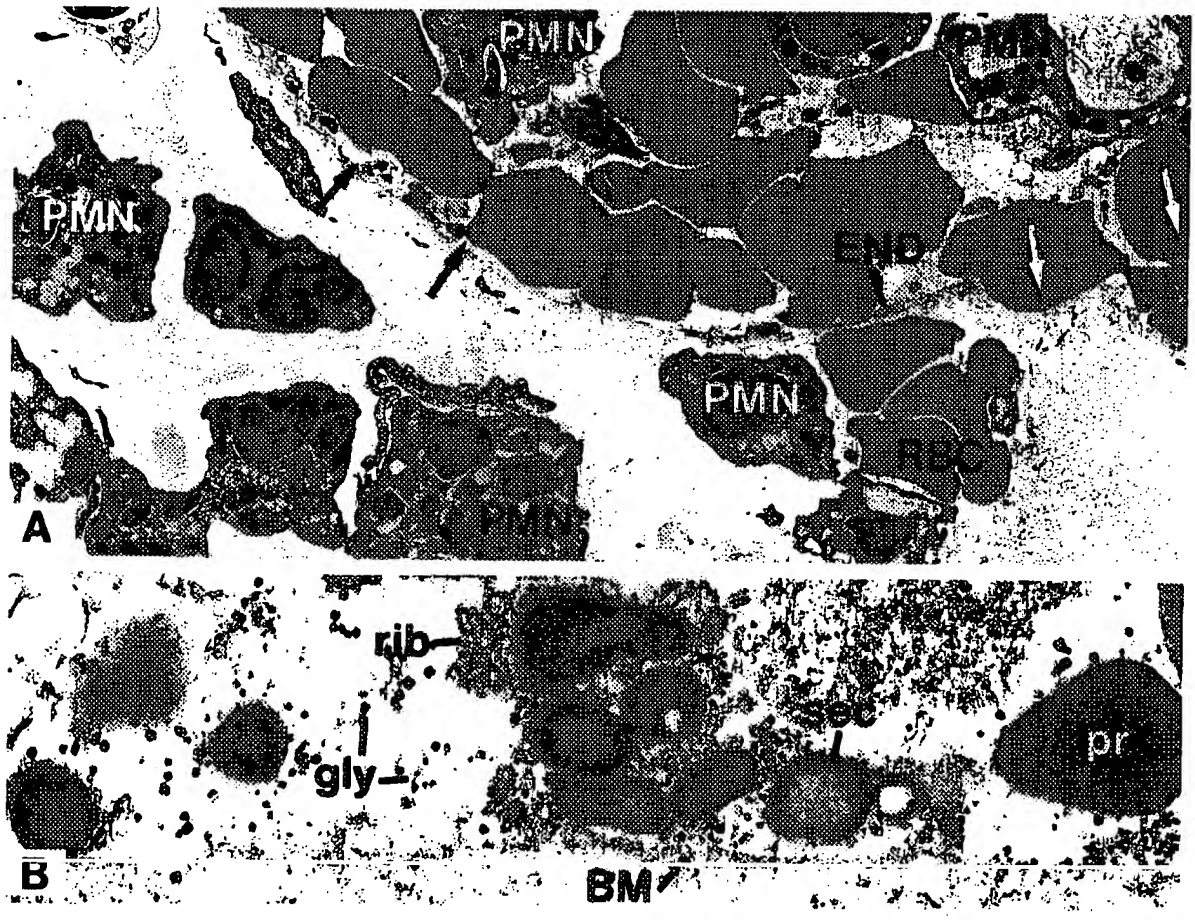


FIG. 5. Example of vessel wall injury. *A*, Most of the lining endothelium is missing. In the *right third* of the illustration, several erythrocytes (*RBC*) are in the perivascular tissue or in the process of crossing the injured vessel wall (*white arrows*). Eight neutrophils (*PMN*) are in the interstitium. *B*, The area between the two *black arrows* in *A*. There are free primary and secondary neutrophil granules (*pr*, *sec*), glycogen (*gly*), and ribosomes (*rib*) in the lumen, above the remnants of the basement membrane (*BM*). *A*, ×3,900; *B*, ×46,000. Reproduced from Movat *et al.*, Am J Pathol 129:463, 1987, Copyright by the American Association of Pathologists, Inc.

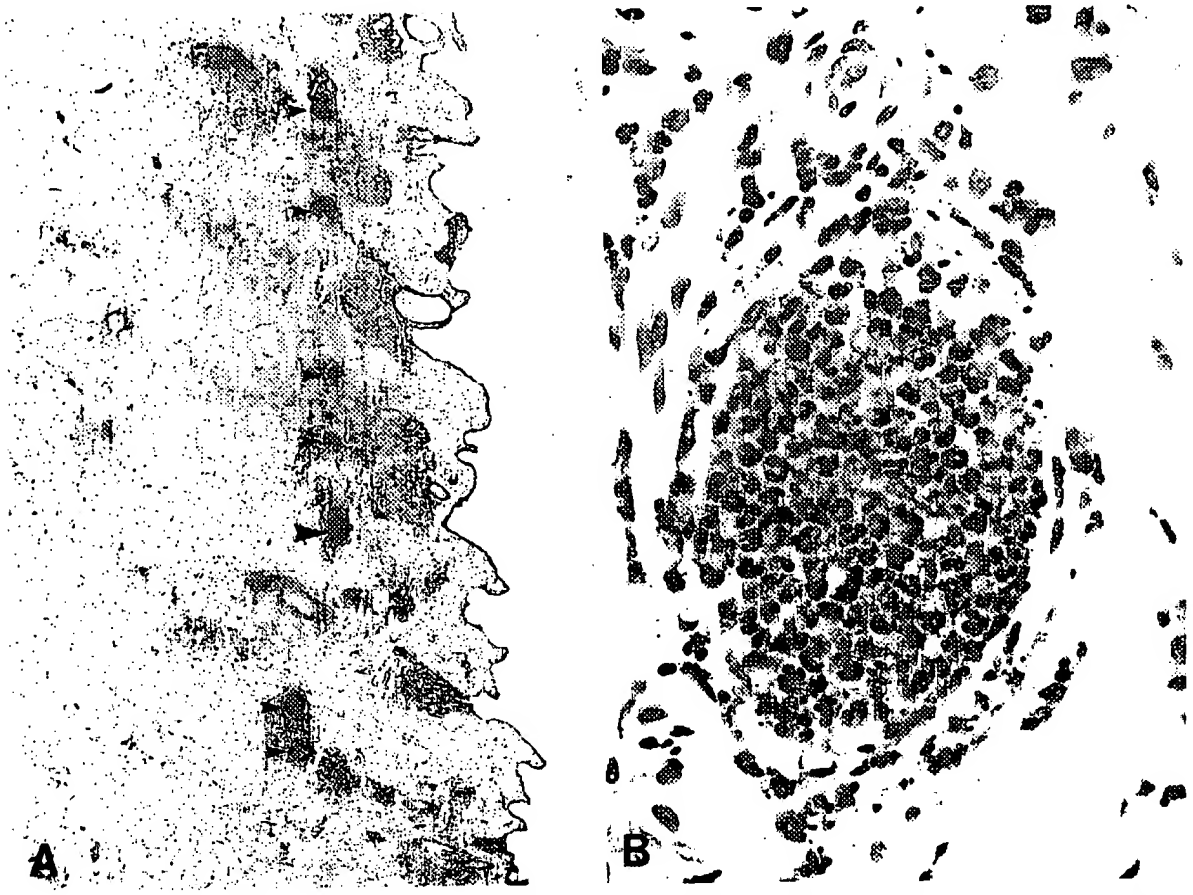


FIG. 6. Shwartzman-like reaction in rabbit skin prepared with intradermal injections of 0.5 μg of IL-1 and 1 μg of TNF. Eighteen hours after the intradermal injections, 10 μg/kg of endotoxin was administered intravenously and the rabbit sacrificed 15 minutes later. *A*, There are numerous venules occluded by microthrombi (small *arrowheads*). *B*, The microthrombi are made up mostly of neutrophils (vessel indicated by large *arrowhead* in *A*). Figure 6*A*, ×35; *B*, ×560.

the induction of a shock-like state with hemorrhagic pulmonary lesions (95). A leukocytopenia and thrombocytopenia were also observed in the last mentioned study and this observation may be important with respect to the intravenous challenging injection in the Shwartzman reaction. The rapid onset and prolonged leukopenia (predominantely neutropenia) observed after endotoxin infusion (29, 109, 110) or injection of *E. coli* (27) may well be mediated by IL-1, or by TNF or by IL-1 acting synergistically with TNF. In keeping with the observations of Okusawa *et al.* (95) on pulmonary changes, when ^{51}Cr-neutrophils were present in the circulation when endotoxin was injected, a large number of these cells were recovered in the lung (29). Furthermore, neutrophils were subsequently mobilized from the bone marrow and many of these ended up in the lungs (30). While the neutropenia after endotoxin or *E. coli* lasted (depending on the dose), from one to several hours, to be able to elicit a protracted shock-like state with leukopenia, Okusawa *et al.* (95) had to follow a bolus (1 μg) by a continuous infusion (5 ng/kg/min each of IL-1 and TNF). Interestingly, when considerably less IL-1 was administered to rats the cytokine was reported to induce only a neutrophilia, whereas TNF induced a neutropenia, followed by two peaks of neutrophilia (the second attributed to intrinsic IL-1 production) (117). There is even a possibility that the intravenous challenge with complement-activating agents may follow a pathway implicating IL-1 and TNF, since *in vitro* C5a and $C5a_{desArg}$ can stimulate blood mononuclear cells to produce IL-1 (94). Potential sources of IL-1 and TNF in the blood are primarily monocytes, and macrophages of the reticular activating system but there is also recent evidence for IL-1 production by neutrophils (111, 122).

INFLAMMATION AND HOST DEFENSE IN GRAM-NEGATIVE INFECTION

With the introduction of antibiotics, Gram-positive infections could be readily controlled, but Gram-negative bacteria became, during the 1950s, problems difficult to deal with, particularly in nosocomial infections (100). Antibiotics have only a limited effect on severe Gram-negative infections such as pneumonia, bacteremia, and septicemia (16, 77). Over a recent 5-year period (1977 to 1981), the mortality in 1,186 episodes of Gram-negative bacteremia was 36.3%, which was no improvement compared with 1924, *i.e.*, the presulfonamide and preantibiotic period (16). Whereas 70% of nosocomial lung infections are attributable to pseudomonas, and 33% to other Gram-negative bacteria, only 5% are caused by

Gram-positive infections (113). Another negative factor is the rapid development of resistance by the bacteria to an antibiotic in hospital-associated infections (102). Sepsis caused by Gram-negative bacteria, alone or in association with other aerobic and anaerobic bacteria, is the leading cause of the syndrome of multiple systems organ failure (4, 14) and is associated with a mortality of 50 to 80%. Should shock occur in association with sepsis, the mortality rate increases to 85 to 90% (105).

A number of bacterial properties contribute to their virulence, including leukocidins, capsules, adhesins, mobility, invasiveness, toxins, iron transport, and serum resistance (15). The diversity of the disease processes due to Gram-negative infection is attributed today mostly to a combination of virulence determinants associated with each bacterial species. For example, only a limited number of *E. coli* strains are usually associated with extraintestinal infections. Urinary tract infections are usually hemolytic (46) and are resistant to the bactericidal action of normal serum (112). One particular class of virulence-associated organelles are known as pili or fimbriae. These hair-like structures foster bacterial adherence to mucosal epithelium via specific receptor-ligand interactions (66). Piliation in *E. coli* permit efficient colonization of the gastrointestinal or genitourinary tracts by enteropathogenic and uropathogenic species respectively (78, 116).

In addition to the above variable factors determining virulence of Gram-negative bacteria, they produce various exotoxins, such as labile and stable enterotoxins (42, 50) and hemolysins (46, 119). However, these microorganisms have one disease-producing factor in common, endotoxin. Whereas the exotoxins are produced and released during bacterial growth, endotoxin is "released" by shedding also from dead bacteria. Release of endotoxin is marked during bacterial lysis (32). Endotoxin or lipopolysaccharide consists of lipid A, a core polysaccharide and the *O*-specific antigen and the biologic activity is known to reside in lipid A. The chemistry of endotoxin was elucidated primarily by Westphal and associates (for reviews see Refs. 40, 120). Endotoxin plays a particularly important role in severe and widespread infections, such as those associated with sepsis and septicemia, in which *E. coli* is the most frequent causative agent (75).

Killed *E. coli* have been shown to shed endotoxin into the medium in which they are suspended (51). Not surprisingly, such bacteria, particularly when deposited in large numbers (20 sites at 6×10^8 bacteria/site) into the dermis, will induce systemic effects attributed to endotoxin, such as fever and neutropenia, followed by neutrophilia (27). Both the magnitude and the duration of the neutropenia were dose-dependent. In these experiments, the number of circulating neutrophils was correlated with the infiltration at the site of *E. coli* injection. Compared with earlier observations where multiple injections of bacteria were administered over a 24-hour period before the quantitation with ^{51}Cr-neutrophils (69), when 20 simultaneous intradermal doses of killed *E. coli* were injected, very few neutrophils emigrated 0 to 4 hours postinjection (during the neutropenic phase). Instead, some influx of neutrophils occurred 6 to 10 hours postinjection (during the neutrophilic phase). When another set of 20 sites was injected 6 hours after the first set of 20 injections (during neutrophilic phase), the rabbits had become refractory to the development of a neutropenia, and a marked accumulation of neutrophils was quantitated in these lesions at 0 to 4 hours (27). The exact roles of various cytokines, and the direct or indirect effects of endotoxin on the levels of circulating neutrophils and their mobilization from the marrow pool remains to be determined.

Using killed *E. coli* has the advantage of fixed and reproducible stimuli. However, within certain dose limits, live *E. coli* elicit essentially similar inflammatory reactions as killed bacteria (86). We extended these studies with live *E. coli*, by comparing the magnitude and kinetics of ^{51}Cr-neutrophil accumulation with the level of circulating neutrophils, the increase in vasopermeability (^{125}I-albumin), and the recovery of bacterial colony-forming units (CFUs) in the dermal lesions of several groups of rabbits: normals, neutropenic (transient or persistent), immunized (active systemic or passive local) (18). Neutrophil emigration and enhanced vasopermeability peaked in 2 to 4-hour-old lesions (2×10^7 or 2×10^8 *E. coli*/site). However, it was essential to elicit at least a partial refractory state to neutropenia by injecting *E. coli* 24 hours before the experiment. This procedure induced a transient mild to moderate neutropenia and a subsequent neutrophilia. With high doses (2×10^{10} *E. coli*/lesion) a protracted neutropenia developed and no neutrophils were delivered to the stimulated sites. Bacterial pour plate colony counts from homogenates of intradermal sites injected with *E. coli*, indicated in normal rabbits early (1 to 2 hours) bacterial multiplication, followed by diminished CFUs at later time points. The decrease in CFUs coincided with the peak emigration of neutrophils into the dermal sites and light and electron microscopy demonstrated phagocytosis of the bacteria by the neutrophils. Animals rendered neutropenic for prolonged periods with nitrogen mustard showed a progressive increase in the numbers of *E. coli* within the intradermal sites, which by 48 hours increased several hundred times over controls. Compared with normals, fewer neutrophils accumulated and there was less plasma exudation. A delayed inflammatory reaction was observed during recovery from neutropenia, as noted already by Issekutz *et al.* (59). As in the lesions with 2×10^{10} *E. coli*/site, in the neutropenic rabbits there was severe dermal necrosis at sites injected with 2×10^7 *E. coli*/site. Animals which were injected with *E. coli* at the time when their circulating neutrophils were recovering from nitrogen mustard treatment, showed only an early (1 to 4 hour) increase in numbers of CFUs, followed by bacterial clearance. Two interesting observations were made in actively immunized animals: no neutropenia developed after the first intradermal injections of *E. coli*, and considerably fewer neutrophils accumulated in the dermis, but interestingly, these were able to cope with bacterial elimination in the same manner as normal controls. Fewer neutrophils accumulated, also with local passive immunization, i.e., when anti-*E. coli* antiserum was deposited at the injection site, but without the capacity to prevent the development of neutropenia.

It was concluded that (a) Neutrophil leukocytes play

a pivotal role in controlling the growth and spread of *E. coli*. (b) In the absence of neutrophils, the bacterial replication is unchecked. (c) In actively immunized rabbits fewer neutrophils can control the infection, (implying that (i) the cellular defense operates more efficiently and (ii) specific antibodies can assist and expedite the clearance of bacteria). (d) Passive transfer of antiserum locally also potentiates the clearance of microorganisms from the site of infection/inflammation. (e) In actively immunized animals no transient neutropenia develops in the circulation.

We propose that cytokines and in particular IL-1 are the principal mediators responsible for mobilizing neutrophils to the inflammatory site where endotoxin is released from Gram-negative bacteria. In immunized animals, fewer neutrophils infiltrated the inflammatory lesions, presumably due to neutralization of endotoxin in the outer wall of the bacteria, leading to less cytokine production, and hence less emigration. Diminished emigration was observed when *E. coli* or endotoxin were treated with antibody or polymyxin B (52). Likewise, when we injected *E. coli* for the first time into actively immunized rabbits, no transient neutropenia developed, probably because the locally liberated endotoxin was bound and this prevented stimulation of IL-1 and TNF synthesis and release. In addition to opsonization, antibody may have another effect in Gram-negative infection, *i.e.*, prevention of a decrease in circulating neutrophils and hence assurance of a constant delivery of neutrophils to the infected site.

Figure 7 illustrates our concept of the inflammatory events which follows the entry of Gram-negative bacteria into tissues. This diagrammatic presentation disregards exotoxins and emphasizes endotoxin and its effects: generation of host-derived inflammatory mediators and their action locally and systemically.

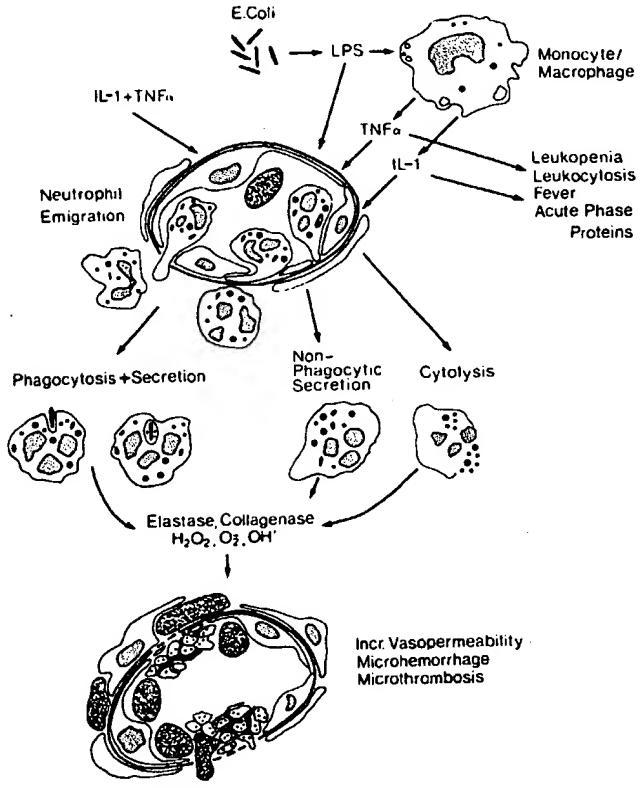


FIG. 7. Diagrammatic representation of events which follow entry of *E. coli* into tissues. The bacteria shed endotoxin (*LPS*) which stimulates monocytes and macrophages (locally and systemically) to synthesize and release IL-1 and TNFα. By acting on the circulating, marginal, and bone marrow pools of neutrophils and their precursors, the cytokines induce neutropenia, followed by neutrophilia (also lymphopenia). These events influence the delivery of neutrophils at the inflammatory site. Other systemic effect of the cytokines are elicitation of fever, acute phase proteins and other components of the acute phase reaction. Through action on the endothelium, IL-1 and TNFα induce neutrophil emigration and accumulation at the site of bacterial multiplication, representing the most important line of defense. IL-1 and TNFα of other sources could also induce neutrophil influx. *In vitro* studies indicate that endotoxin could act directly on the endothelium to induce synthesis and release of IL-1. The emigrated neutrophils release lysosomal enzymes and oxygen radicals by cytolysis, phagocytosis of the bacteria, and secretion and perhaps by nonphagocytic secretion. The severely injured microvessels exhibit increase in vasopermeability, hemorrhage, and thrombosis.

This work was supported by grants from the Medical Research Council of Canada (MT-1251) and the Heart and Stroke Foundation of Ontario (T082).

Dr. Cybulsky is the recipient of a Medical Research Council of Canada Research Fellowship; present address: Department of Pathology, Vascular Research Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115.

Dr. Movat is a Career Investigator of the Medical Research Council of Canada.

Address reprint requests to: Henry Z. Movat. M.D., Department of Pathology, University of Toronto, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8 Canada.

REFERENCES

1. Anderson DC, Springer TA: Leukocyte adhesion deficiency: an inherited defect in the Mac-1 LFA-1, and p150, 95 glycoprotein. Annu Rev Med 38:175, 1987
1a. Arfors K-E, Lundberg C, Lindbom L, Lundberg K, Beatty PG, Harlan JM: A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage *in vivo*. Blood 69:338, 1987
2. Atkins E, Bodel P, Francis L: Release of an endogenous pyrogen *in vitro* from rabbit mononuclear cells. J Exp Med 126:357, 1967
3. Averbrook B, Ulitch T, Jeffes E, Yamato R, Chow G, Masunaka I, Granger G: Human alpha lymphotoxin and TNF induce different types of inflammatory responses in normal tissues (abstr). Fed Proc 46:562, 1987
4. Baue AE, Chaudry IH: Prevention of multiple systems failure. Surg Clin North Am 60:1167, 1980
5. Beck C, Habict GS, Benach JL, Miller F: Interleukin 1: A common endogenous mediator of inflammation and the local Shwartzman reaction. J Immunol 136:3025, 1986
6. Beutler B, Cerami AC: Cachectin. More than a tumor necrosis factor. N Engl J Med 3156:379, 1987
7. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effects of endotoxin. Science 229:869, 1985
8. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr: Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin. Proc Natl Acad Sci USA 83:4533, 1986
9. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone, MA Jr: Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cell. J Exp Med 160:681, 1984
10. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr: Identification of an inducible endothelial-leukocyte adhesion molecule. PNAS 84:9238, 1987
11. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone

MA Jr: Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. J Clin Invest 76:2003, 1985
12. Bevilacqua MP, Scheef RR, Gimbrone MA Jr, Loskutoff DJ: Regulation of the fibrinolytic system of cultured vascular endothelium by interleukin 1. J Clin Invest 78:587, 1986
13. Bonny RJ, Humes JL: Physiological and pharmacological regulation of prostaglandin and leukotriene production by macrophages. J Leukocyte Biol 35:1, 1984
14. Borzotta AP, Polk HC Jr: Multiple systems organ failure. Surg Clin North Am 63:315, 1983
15. Brubaker RR: Mechanisms of bacterial virulence. Annu Rev Microbiol 39:21, 1985
16. Bryan CS, Reynolds KL, Brenner ER: Analysis of 1,186 episodes of gram-negative bacteremia in non-university hospitals: the effect of antimicrobial therapy. Rev Infect Dis 5:629, 1983
17. Cannon JG, Dinarello CA: Increased plasma interleukin-1 activity in women after ovulation. Science 227:1247, 1985
18. Chan W, Movat HZ: Microbicidal role of inflammation in Gram negative infection (abstr). Fed Proc, in press 1988
19. Cochrane CG, Janoff A: The Arthus reaction: a model of neutrophil and complement-mediated injury. In The Inflammatory Process, Vol 3, edited by Zweifach BS, Grant L, McCluskey RT, p 85. New York, Academic Press, 1974
20. Colditz IG, Movat HZ: Chemotactic factor-specific desensitization of skin to infiltration by polymorphonuclear leukocytes. Immunol Lett 8:83, 1984
21. Colditz IG, Movat HA: Desensitization of acute inflammatory lesions to chemotaxins and endotoxin. J Immunol 133:2163, 1984
22. Colditz IG, Movat HZ: Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. J Immunol 133:2169, 1984
23. Cotran RS, Gimbrone MA Jr, Bevilacqua MP, Mendrick DL, Pober JS: Induction and detection of a human endothelial activation antigen *in vivo*. J Exp Med 164:661, 1986
24. Crawford JP, Movat HZ, Minta JO, Opas M: Acute inflammation induced by immune complexes in the microcirculation. Exp Mol Pathol 42:175, 1985
25. Cybulsky MI, Colditz IG, Movat HZ: Interleukin 1 activity in the local recruitment of PMNs. Its potential role in endotoxin-induced acute inflammation (abstr). Fed Proc 44:1260, 1985
26. Cybulsky MI, Colditz IG, Movat HZ: The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. Am J Pathol 124:6, 1986
27. Cybulsky MI, Cybulsky IJ, Movat HZ: Neutropenic responses to intradermal injections of *Escherichia coli*. Effects on the kinetics of polymorphonuclear leukocyte emigration. Am J Pathol 124:1, 1986
28. Cybulsky MI, McComb DJ, Dinarello CA, Movat HZ: Mediation by interleukin-1 of neutrophil leukocyte emigration induced by endotoxin. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 38. Basel, S Karger AG, 1987
28a. Cybulsky MI, McComb DJ, Movat HZ: Neutrophil leukocyte emigration induced by endotoxin: mediator roles of IL-1 and tumor necrosis factor α. J Immunol, in press 1988
29. Cybulsky MI, Movat HZ: Experimental bacterial pneumonia in rabbits: polymorphonucler leukocyte margination and sequestration in rabbit lungs and quantitation and kinetics of ^{51}Cr-labelled polymorphonuclear leukocytes in *E. coli*-induced lung lesions. Exp Lung Res 4:47, 1982
30. Cybulsky MI, Movat HZ: Application of ^{51}Cr-labelled PMN leukocytes in quantitating PMN kinetics in systemic and local inflammatory mediated processes. Effects of endotoxin, complement and interleukin 1. Surv Synth Pathol Res 1:208, 1983
31. Cybulsky MI, Movat HZ, Dinarello CA: Role of interleukin 1 and tumor necrosis factor α in acute inflammation. Ann Inst Pasteur, (Paris), 138:505, 1987
32. De Voe IW: Ingestion of degraded meningococci by polymorphonuclear leukocytes. J Bacteriol 125:258, 1976
33. Dinarello CA: Interleukin-1. Rev Infect Dis 6:51, 1984
34. Dinarello CA: Interleukin-1. Amino acid sequence, multiple biological activities and comparison with tumor necrosis factor (cachectin). Year Immunol 2:68, 1986
35. Dinarello CA, Cannon JC, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, O'Connor JV: Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 163:1433, 1986
36. Dinarello CA, Ikejina T, Warner SJC, Orecole SF, Lonnemann G, Cannon JG, Libby P: Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits *in vivo* in human mononuclear cells *in vitro*. J Immunol 139:1902, 1987
37. Dunn CJ, Fleming WE: The role of interleukin-1 in the inflammatory response with particular reference to endothelial cell-leukocyte adhesion. In The Physiologic, Metabolic, and Immunologic Actions of Interleukin-1, edited by Kluger MJ, Oppenheim JJ, Powanda MC, p 45. New York, Alan R Liss, 1985
38. Elias RM, Johnston MG, Hayashi A, Nelson W: Decreased lymphatic pumping after intravenous endotoxin administration in sheep. Am J Physiol 253:183, 1987
39. Gahring L, Baltz M, Pepys MB, Daynes R: Effect of ultraviolet radiation on production of endothelial cell thymocyte-activating factor/interleukin 1 *in vivo* and *in vitro*. Proc Natl Acad Sci USA 81:1198, 1984
40. Galanos CO, Lüderitz O, Rietschel ET, Wesphal O: Newer aspects of the chemistry and biology of bacterial lipopolysaccharides, with special reference to their lipid A component. In Biochemistry of Lipids II, edited by Goodwin TW, p 239. Baltimore MD, University Park Press, 1977
41. Gamble RJ, Harlan JM, Klebanoff SJ, Lopez AF, Vadas MA: Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 82:8667, 1985
42. Gemmell CG: Comparative studies of the nature and biological activities of bacterial enterotoxins. J Med Microbiol 17:217, 1984
43. Goerd S, Zwadlo G, Schlegel R, Hagemeier H-H, Sorg C: Characterization and expression kinetics of an endothelial cell activation antigen present in vivo only in acute inflammatory tissues. Exp Cell Biol 55:117, 1987
44. Grantstein RD, Margolis R, Mizel SB, Sauder DN: *In vivo* inflammatory activity of epidermal cell-derived thymocyte-activating factor and recombinant interleukin 1 in the mouse. J Clin Invest 77:120, 1986
45. Habicht GS, Beck G: IL-1 is an endogenous mediator of acute inflammation and of the local Shwartzman reaction. In The Physiologic, Metabolic, and Immunologic Actions of Interleukin-1, edited by Kluger MJ, Oppenheim JJ, Powanda MC, p 13. New York, Alan R Liss, 1985
46. Hacker J, Hughes C, Hof H, Goebel W: Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. Infect Immunol 42:57, 1983
47. Hamilton SM, Johnston MG, Fong A, Pepevnak C, Semple JL, Movat HZ: Relationship between increased vascular permeability and extravascular albumin clearance in rabbit inflammatory responses induced with *Escherichia coli*. Lab Invest 55:580, 1986
48. Hanson DF, Murphy PA, Windle BE: Failure of rabbit neutrophils to secrete endogenous pyrogen when stimulated with staphylococci. J Exp Med 151:1360, 1980
49. Hay JB, Johnston MG, Hobbs BB, Movat HZ: The use of radioactive microspheres to quantitate hyperemia in dermal inflammatory sites. Proc Soc Exp Biol Med 150:641, 1975
50. Holmgren J: Toxins affecting intestinal transport processes. In The Virulence of *Escherichia coli*, edited by Sussman M, p 177. London, Academic Press, 1985
51. Issekutz AC, Bhimji S: Role of endotoxin in the leukocyte infiltration accompanying *Escherichia coli* inflammation. Infect Immun 36:558, 1982
52. Issekutz AC, Bhimji S, Bertolussi R: Effect of immune serum or polymyxin B on *Escherichia coli*-induced inflammation and vascular injury. Infect Immun 36:548, 1982
53. Issekutz AC, Megyeri P: Induction of leukocyte infiltration by endotoxin. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 24. Basel, S Karger AG, 1987
54. Issekutz AC, Megyeri P, Issekutz TB: Role of macrophage products in endotoxin-induced polymorphonuclear leukocyte accumulation during inflammation. Lab Invest 56:49, 1987
55. Issekutz AC, Movat HZ: Quantitation of neutrophil infiltration *in vivo*. Immunol Lett 1:27, 1979
56. Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of rabbit neutrophil leukocyte accumulation in the skin in response

to chemotactic agents and *Escherichia coli*. Lab Invest 42:310, 1980
57. Issekutz AC, Movat HZ: The effect of vasodilatory prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. Am J Pathol 107:300, 1982
58. Issekutz AC, Movat KW, Movat HZ: Enhancement of vascular permeability and haemorrhage-inducing activity of rabbit $C5a_{desArg}$: probable role of polymorphonuclear leukocyte lysosomes. Clin Exp Immunol 41:512, 1980
59. Issekutz AC, Ripley M, Rochon Y, Pi-Jimenez E, Wright B: A role for hemolysin in *Escherichia coli*-induced inflammation in granulocytopenic rabbits. J Infect Dis 150:925, 1984
60. Issekutz AC, Wankowicz Z: Synergy between tumor necrosis factor (TNFα) and interleukin 1 (IL-1) in the induction of polymorphonuclear leukocyte emigration (abstr). Fed Proc 46:737, 1987
61. Issekutz TB, Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of monocyte migration into acute inflammatory tissue. Am J Pathol 103:74, 1981
62. Jeynes BJ, Issekutz AC, Issekutz TB, Movat HZ: Quantitation of platelets in the microcirculation. Measurement of indium-111 in microthrombi induced in rabbits by inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 165:445, 1980
63. Johnston MG: Interaction of inflammatory mediators with the lymphatic vessel. Pathol Immunopathol Res 6:177, 1987
64. Johnston MG, Hay JB, Movat HZ: Kinetics of prostaglandin production in various inflammatory lesions, measured in draining lymph. Am J Pathol 95:225, 1979
65. Kampschmidt RF: Leukocytic endogenous mediator/endogenous pyrogen. In Infection: The Physiologic and Metabolic Responses of the Host, edited by Powanda MC, Canonico PG, p 403. Amsterdam, Elsevier Science Publisher, 1981
66. Klemm P: Fimbrial adhesions of *Escherichia coli*. Rev Infect Dis 7:321, 1985
67. Koj A: Biologic functions of acute-phase proteins. In The Acute-Phase Response to Injury and Infection, edited by Gordon AH, Koj A, p 145. Amsterdam, Elsevier, 1985
68. Kopaniak MM, Issekutz AC, Burrowes CE, Movat HZ: The quantitation of hemorrhage in the skin: measurement of hemorrhage in the microcirculation in inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 163:126, 1980
69. Kopaniak MM, Issekutz AC, Movat HZ: Kinetics of acute inflammation induced by *E. coli* in rabbits. Quantitation of blood flow, enhanced vascular permeability, hemorrhage and leukocyte accumulation. Am J Pathol 98:485, 1980
70. Kopaniak MM, Movat HZ: Kinetics of acute inflammation induced by *Escherichia coli* in rabbits. II. The effect of hyperimmunization, complement depletion, and depletion of leukocytes. Am J Pathol 110:13, 1983
71. Libby P, Ordovas JM, Auger KR, Robbin HH, Biriny LK, Dinarello CA: Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult vascular endothelial cells. Am J Pathol 124:179, 1986
72. Libby P, Ordovas JM, Birinyi LK, Auger KR, Dinarello CA: Inducible interleukin 1 expression in human vascular smooth muscle cells. J Clin Invest 78:1432, 1986
73. Luscinskas FW, Bevilacqua MP, Brock AF, Arnaout MA, Gimbrone MA Jr: Endothelial-leukocyte adhesion: Contributions of endothelial dependent and leukocyte-dependent mechanisms (abstr). Fed Proc, in press 1988
74. Marasco WA, Phan SH, Krutsch H, Showell HJ, Feltner DE, Nairn R, Becker EL, Ward PA: Purification and identification of formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J Biol Chem 259:5430, 1984
75. McCabe WR: Gram negative bacteremia. In Infectious Diseases and Medical Microbiology, edited by Braude AI, Davis CE, Fierer J, p 1177. Philadelphia, WB Saunders, 1986
76. McComb DJ, Cybulsky MI, Movat HZ: PMN emigration: protein synthesis dependent and independent mechanisms (abstr). Fed Proc 46:1390, 1987
77. McGowan JE, Barnes MW, Finland M: Bacteremia at Boston City Hospital: occurrence and mortality during 12 selected years (1935–72), with special reference to hospital-acquired cases. J Infect Dis 132:316, 1975
78. Mooi FR, De Graaf FK: Molecular biology of fimbriae of enterotoxigenic *Escherichia coli*. Curr Topics Microbiol Immunol 118:119, 1985
79. Morrison DC, Ulevitch RJ: The effects of bacterial endotoxins on host mediation systems. Am J Pathol 93:526, 1978
80. Movat HZ: Microcirculation in disseminated intravascular coagulation induced by endotoxin. In Handbook of Physiology, Vol IV, The Cardiovascular System, edited by Renkin EN, Michel CC, p 1047. Bethesda MD, American Physiological Society, 1984
81. Movat HZ: The Inflammatory Reaction, Amsterdam, Elsevier, 1985
82. Movat HZ, Burrowes CE: Elicitation of the Shwartzman reaction by a combination of endotoxin and agents which activate the complement system: microvascular events. In Immunopharmacology of Endotoxicosis, edited by Agarwal MK, Yoshida M, p 197. Berlin/New York, Walter de Gruyter and Co, 1984
83. Movat HZ, Burrowes CE: Local Shwartzman reaction: endotoxin-mediated inflammatory and thrombo-hemorrhagic lesions. In Handbook of Endotoxin, Volume 3, Cellular Biology of Endotoxin, edited by Berry LJ, p 260. Amsterdam, Elsevier, 1985
84. Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA: Role of complement, interleukin-1 and tumor necrosis factor in a local Shwartzman-like reaction. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 69. Basel, S Karger AG, 1987
85. Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA: Acute inflammation and a Shwartzman-like reaction induced by interleukin 1 and tumor necrosis factor. Synergistic action of the cytokines in the induction of inflammatory and microvascular injury. Am J Pathol 129:463, 1987
86. Movat HZ, Cybulsky MI, Colditz IG, Chan MKW, Dinarello CA: Acute inflammation in gram negative infection. Role of endotoxin, interleukin 1, tumor necrosis factor and neutrophils. Fed Proc 46:97, 1987
87. Movat HZ, Jeynes BJ, Wasi S, Movat KW, Kopaniak MM: Quantitation of the development and progression of the local Shwartzman reaction. In Bacterial Endotoxins and Host Response, edited by Agarwal MK, p 179. Amsterdam, Elsevier, 1980
88. Movat HZ, Rettl C, Burrowes CE, Johnston MG: The *in vivo* effect of leukotriene B_4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement ($C5a_{desArg}$) and enhancement by prostaglandin E_2. Am J Pathol 115:233, 1984
89. Movat HZ, Wasi S: Severe microvascular injury induced by lysosomal releasates of human polymorphonuclear leukocytes. Increase in vasopermeability, hemorrhage, and microthrombosis due to degradation of subendothelial and perivascular matrices. Am J Pathol 121:404, 1985
90. Müller-Berghaus G, Lasch H-G: Microcirculatory disturbances induced by generalized intravascular coagulation. In Handbook of Experimental Pharmacology, Vol 16, Experimental Production of Diseases: Heart and Circulation, edited by Schmier J, Eichler O, p 429. Berlin, Springer-Verlag, 1975
91. Nachman RL, Hajjar KA, Silverstein RL, Dinarello CA: Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. J Exp Med 163:1260, 1986
92. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D: Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363, 1986
93. Nawroth PP, Stern DM: Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 163:740, 1986
94. Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank MM, Burke JF, Gelfand JA: C5a induction of interleukin 1. Synergistic effect with endotoxin or interferon-γ. J Immunol 139:2635, 1987
95. Okusawa S, Gelfand JA, Ikejima T, Connolly RJ, Dinarello CA: Interleukin-1 induces a shock-like state in rabbits: synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J Clin Invest, in press 1988
96. Parry SH, Rooke DM: Adhesions and colonization factors of *Escherichia coli*. In The Virulence of *Escherichia coli*, edited by Sussman M, p 79. London, Academic Press, 1985
96a. Pettipher ER, Higgs GA, Henderson B: Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in

the synovial joint. Proc Natl Acad Sci USA 83:8749, 1986
97. Pober JS, Bevilacqua MP, Mendrick DL, Lapierre LA, Fiers W, Gimbrone MA Jr: Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J Immunol 136:1680, 1986
98. Pober JS, Lapierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA Jr: Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. J Immunol 138:3319, 1987
99. Pohlman TH, Stanness KA, Beatty PG, Ochs HD, Harlan JM: An endothelial cell surface factor (s) induced *in vitro* by lipopolysaccharide, interleukin 1 and tumor necrosis factor-increases neutrophil adherence by a CDw18-dependent mechanisms. J Immunol 136:4548, 1986
100. Rogers DE: The changing pattern of life-threatening microbial disease. N Engl J Med 261:677, 1959
101. Ruff MR, Gifford GE: Tumor necrosis factor. Lymphokines 2:235, 1981
102. Sanders CC, Sanders WE Jr: Microbial resistance to newer generation β-lactam antibiotics: clinical and laboratory implications. J Infect Dis 151:399, 1981
103. Schleimer RP, Rutledge BK: Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin and tumor-promoting phorbol diesters. J Immunol 136:649, 1986
104. Shwartzman G: Phenomenon of Local Tissue Reactivity and its Immunological, Pathological and Clinical Significance, New York, Paul Hoeber, 1937
105. Sinanan M, Maier RV, Corrico J: Labarotomy for intra-abdominal sepsis in an intensive care unit. Arch Surg 119:652, 1985
106. Springer TA, Dustin ML, Kishimoto TK, Marlin SD: Lymphocyte function-associated LFA-1, CD2, and LFA-3 receptors of the immune system. Annu Rev Immunol 5:223, 1987
107. Stern DM, Bank I, Nawroth PP, Cassimeris J, Kisiel W, Fenton JW II, Dinarello C, Chess L, Jaffe EA: Self-regulation of procoagulant events on the endothelial cell surface. J Exp Med 162:1223, 1985
108. Stern D, Nawroth P, Handley D, Kisiel W: An endothelial cell-dependent pathway of coagulation. Proc Natl Acad Sci USA 82:2523, 1985
109. Stetson CA Jr: Similarities in the mechanisms determining the Arthus and Shwartzman phenomenon. J Exp Med 94:347, 1951
110. Stetson CA Jr, Good RA: Studies on the mechanisms of the Shwartzman phenomenon: Evidence for the participation of polymorphonuclear leukocytes in the phenomenon. J Exp Med 93:49, 1951
111. Tiku K, Tiku ML, Skosey JL: Interleukin 1 production by human polymorphonuclear leukocytes. J Immunol 136:3677, 1986
112. Timmis KN, Boulnois GJ, Bitter-Sauermann D, Cabello FC: Surface components of *Escherichia coli* that mediate resistance to the bactericidal activities of serum and phagocytes. Curr Top Microbiol Immunol 118:197, 1985
113. Tobin MK, Grenvik A: Nosocomial lung infection and its diagnosis. Crit Care Med 12:191, 1984
114. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark JW, Hariri RJ, Fahey III TJ, Zentella A, Albert JD, Shires GT, Cerami A: Shock and tissue injury induced by recombinant human cachectin. Science 234:470, 1986
115. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Exp Biol Med 133:1384, 1970
116. Uhlin BE, Baga M, Goranson M, Lindberg FP, Lund B, Normark S: Genes determining adhesin formation in uropathogenic *Escherichia coli*. Curr Top Microbiol Immunol 118:163, 1985
117. Ulich TR, del Castillo J, Keys M, Granger GA, Ni R-X: Kinetics and mechanisms of recombinant human interleukin 1 and tumor necrosis factor-α-induced changes in circulating numbers of neutrophils and lymphocytes. J Immunol 139:3406, 1987
118. Warner SJC, Auger KR, Libby P: Interleukin 1 induces interleukin 1. II. Recombinant human interleukin 1 induces interleukin 1 production by adult human vascular endothelial cells. J Immunol 139:1911, 1987
119. Welch RA, Falkow S: Characterization of *Escherichia coli* hemolysins conferring quantitative differences in virulence. Infect Immun 43:156, 1984
120. Westphal O, Jann K, Himmelspach K: Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. Prog Allergy 33:9, 1983
121. Windle BE, Murphy PA, Cooperman S: Rabbit polymorphonuclear leukocytes do not secrete endogenous pyrogen or interleukin 1 when stimulated by endotoxin, polyinosine; polycytosine, or muramyl dipeptide. Infect Immunol 39:1142, 1983
122. Yoshinaga M, Goto F, Goto K, Ohkawara S, Kitamura M, Mori S: Triggering of polymorphonuclear leukocytes to produce interleukin-1 at the inflammatory site. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 169. Basel, S Karger AG, 1987

STIC-ILL

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

THE MOUSE/HUMAN CHIMERIC MONOCLONAL ANTIBODY cA2 NEUTRALIZES TNF IN VITRO AND PROTECTS TRANSGENIC MICE FROM CACHEXIA AND TNF LETHALITY IN VIVO

Scott A. Siegel,[1*] David J. Shealy,[1] Marian T. Nakada,[2] Junming Le,[3] Donna S. Woulfe,[2†] Lesley Probert,[4] George Kollias,[4] John Ghrayeb,[2] Jan Vilcek,[3] Peter E. Daddona[1‡]

The pleiotropic cytokine tumour necrosis factor-α (TNF) is thought to play a central role in infectious, inflammatory and autoimmune diseases. Critical to the understanding and management of TNF-associated pathology is the development of highly specific agents capable of modifying TNF activity. We evaluated the ability of a high affinity mouse/human chimeric anti-TNF monoclonal antibody (cA2) to neutralize the in vitro and in vivo biological effects of TNF. cA2 inhibited TNF-induced mitogenesis and IL-6 secretion by human fibroblasts, TNF-priming of human neutrophils, and the stimulation of human umbilical vein endothelial cells by TNF as measured by the expression of E-selectin, ICAM-1 and procoagulant activity. cA2 also specifically blocked TNF-induced adherence of human neutrophils to an endothelial cell monolayer. Receptor binding studies suggested that neutralization resulted from cA2 blocking of TNF binding to both p55 and p75 TNF receptors on the cells. In vivo, repeated administration of cA2 to transgenic mice that constitutively express human TNF reversed the cachectic phenotype and prevented subsequent mortality. These results demonstrated that cA2 effectively neutralized a broad range of TNF biological activities both in vitro and in vivo.

Tumour necrosis factor-α (TNF) is a cytokine that exhibits a pleiotropic spectrum of activities, with receptors found on virtually all cell types examined.[1,2] The natural functions of TNF are thought to include modulation of the host immune and inflammatory response to a variety of infectious, malignant and autoimmune conditions as part of a complex regulatory mechanism in which numerous other cytokines participate.[3] While initial TNF expression in response to infection or injury would be considered beneficial, excessive production, usually by activated monocytes and macrophages, can result in significant pathological changes.

From The Departments of [1]Immunology and [2]Molecular Biology, Centocor, Inc., 200 Great Valley Parkway, Malvern PA 19355, USA; [3]Department of Microbiology, New York University Medical Center, 550 First Ave., New York, NY 10016, USA; and [4]Laboratory of Molecular Genetics, Hellenic Pasteur Institute, 127 Av. Vas. Sofias, Athens 115 21, Greece.

Present address: *Phytera, Inc., 377 Plantation Street, Worcester, MA 01605; †University of Pennsylvania, Department of Pharmacology, Philadelphia, PA 19104; ‡Alza Corp., 950 Page Mill Road, Palo Alto, CA 94303, USA.

Correspondence to: Dr David Shealy, Centocor, Inc., 200 Great Valley Parkway, Malvern, PA 19355, USA.

Received 4 February 1994; revised and accepted for publication 7 June 1994

1043-4666/95/010015+11 $08.00/0

KEY WORDS: TNF-α/cA2/Rheumatoid arthritis

TNF has been implicated as the primary mediator in bacterial sepsis since it is the first proinflammatory cytokine detected in primate and human volunteer studies where serum cytokine levels were measured after administration of endotoxin.[4] Administration of TNF to rodents and dogs induced a profile of pathophysiological changes and lethality similar to that seen after endotoxin challenge.[5,6] Neutralizing antibodies to TNF have been shown to prevent physiological changes and death in animal models of endotoxin and bacteremia.[7-9]

There are also a number of autoimmune disorders in which TNF appears to play a significant role,[10-12] but the evidence is most persuasive in rheumatoid arthritis.[13-16] Rheumatoid arthritis (RA) is characterized by a chronic inflammation of the synovial lining of multiple joints. Synovial cells proliferate along with infiltrating inflammatory cells and vascularity increases markedly. Ultimately, the release of degradative enzymes results in irreversible erosion of the bone and cartilage components of the joint.[13,14] Levels of TNF are not only elevated in synovial fluid from the joints of RA patients[17,18] but cells from the synovial fluid continue to produce TNF when cultured in vitro.[19] Perhaps the most direct evidence that TNF plays a pivotal role in the development of arthritis was obtained by the constitutive expression of TNF in transgenic mice.[20] Such mice develop chronic inflammatory polyarthritis at a specific age (depending on the transgenic mouse line)

and with a 100% phenotypic penetrance. Thus, TNF appears to be an attractive target and an antibody which efficiently neutralizes human TNF may be an effective treatment in RA.[20,21]

We have previously described the construction of a chimeric mouse/human IgG_1 monoclonal antibody which binds to TNF.[22] This antibody, designated cA2, exhibited high affinity and specificity for TNF and neutralized both recombinant and natural human TNF in the standard assay of TNF cytotoxicity. cA2 was also shown to be highly species-specific, neutralizing TNF from only humans and chimpanzees. The safety and potential efficacy of cA2 in treating autoimmune disorders is currently being evaluated in human clinical trials.[23,24]

In this study we examined the effect of cA2 on the in vitro biological activity of TNF and on TNF-receptor interactions. The ability of cA2 to modulate the in vivo activity of TNF was also investigated using an established transgenic mouse line that develops a cachectic phenotype and accelerated mortality due to constitutive expression of human TNF.[25]

RESULTS

Effect of cA2 on TNF-stimulated FS-4 fibroblasts

The fibroblast cell line FS-4 proliferates and secretes IL-6 in response to recombinant human TNF-α (TNF).[26,27] As shown in Table 1, TNF at concentrations ranging from 0.3 to 7.5 ng/mL induced FS-4 fibroblasts to produce IL-6 levels ranging from 1300 to 2500 pg/mL. When cA2 was added to the medium at the same time as TNF, the induction of IL-6 was completely blocked at the 0.3 and 1.5 ng/mL TNF dose levels, and only a small amount of IL-6 was detected (300 pg/mL) at the highest (7.5 ng/mL) TNF dose. The control antibody had no effect.

Similarly, cA2 was shown to block the mitogenic effect of TNF on FS-4 fibroblasts (Table 2). When cA2 was added to the culture medium with TNF, proliferation was blocked at all three TNF levels tested (0.1, 0.5 and 2 ng/mL). The control IgG had no effect on the TNF-induced proliferation of FS-4 fibroblasts. A significant difference ($P \leq 0.03$ at all concentrations) was demonstrated between the cA2 and control groups using the nonparametric Wilcoxon test. In additional experiments, cA2 also completely inhibited mitogenesis at TNF concentrations up to 8 ng/mL (data not shown).

Effect of cA2 on TNF-stimulated human umbilical vein endothelial (HUVE) cells

HUVE cells produce a procoagulant activity (PCA) when exposed to TNF which appears to be related to

TABLE 1. Neutralization of TNF-induced IL-6 secretion by fibroblasts

	IL-6 production (pg/mL)			
Antibody	No TNF	0.3 ng/mL TNF	1.5 ng/mL TNF	7.5 ng/mL TNF
None	<200	1360	2000	2560
Control	<200	1600	1960	2160
cA2	<200	<200	<200	300

Recombinant human TNF, preincubated with or without 4 μg/mL cA2 or control antibody, was added to cultures of FS-4 human fibroblasts. After 18 h incubation, IL-6 levels in the supernatant were determined by immunoassay.

TABLE 2. Neutralization of TNF-induced mitogenesis in fibroblast cultures

	Cell Density (OD 630 nm × 10^3)			
Antibody	No TNF	0.1 ng/mL TNF	0.5 ng/mL TNF	2.0 ng/mL TNF
None	36 ± 1	50 ± 1	55 ± 2	63 ± 1
Control	35 ± 2	49 ± 1	54 ± 1	63 ± 1
cA2	39 ± 1	37 ± 1	39 ± 1	41 ± 2

FS-4 human fibroblasts were seeded in 96-well plates. Recombinant human TNF, preincubated with or without 4 μg/mL cA2 or control antibody, was added to the cultures and cell density was determined by staining 5 days later. Data represent the mean (± standard deviation) of quintuplicate wells.

tissue factor upregulation.[28] After the cells are lysed by freezing and thawing, PCA can be detected by measuring the clotting time of plasma to which calcium and cell lysate have been added. Figure 1 shows that while a clotting time of about 130 sec is obtained using an unstimulated HUVE lysate, addition of lysate from HUVE cells exposed to 25 ng/mL TNF for 4 h at 37°C shortens the clotting time by 50%. This reduction in clotting time was blocked by the addition of cA2 in the HUVE cell medium during the time of TNF exposure. A final cA2 concentration of 0.37 µg/mL could completely neutralize TNF-induced PCA while isotype-matched control IgG at 10 µg/mL had no effect.

TNF also acts on HUVE cells by inducing the cell surface expression of the adhesion proteins E-selectin[29] and ICAM-1.[30] Peak temporal expression of these surface antigens varies, however they can be individually quantified using specific monoclonal antibodies. The observed levels of TNF-induced E-selectin and ICAM-1 expression on the HUVE cell surface were reduced in a dose-dependent manner by the inclusion of cA2, while control IgG had no effect (Fig. 2). Induction of peak E-selectin expression (measured at 4 h) was fully abrogated by 0.5 µg/mL of cA2 (Fig. 2A), and complete blocking of peak ICAM-1 expression (at 23 h) required 0.1 µg/mL of cA2 (Fig. 2B).

In vivo, the expression of E-selectin and ICAM-1 by endothelial cells exposed to TNF results in the binding of circulating neutrophils and eventual extravasation into the surrounding tissues.[31] In order to determine if cA2 could effectively block the adhesion of neutrophils in an in vitro system, HUVE cell monolayers were first exposed to TNF in the presence and absence of cA2 for 4 h, then exposed to isolated human neutrophils. After washing, the percentage of neutrophils that adhered to the HUVE cells was determined using a neutrophil-specific myeloperoxidase assay. When treated with TNF alone, neutrophil adherence increased 52%, a fourfold increase over unstimulated HUVE cells (Fig. 3). Increasing amounts of cA2 reduced the degree of neutrophil adherence in a dose-dependent manner, while 3.3 µg/mL of control IgG had no effect. cA2 had no effect on neutrophil adherence to HUVE cells treated with IL-1 or LPS, demonstrating that the inhibitory effect of cA2 is directed specifically at TNF.

The inhibition of TNF-induced neutrophil adherence was also apparent by phase contrast light microscopy (Fig. 4). Neutrophils can be identified by their rounded, highly refractile appearance as opposed to the flat morphology exhibited by the HUVE cell monolayer. In Panel A, unstimulated HUVE cells show few adherent neutrophils compared to HUVE cells stimulated with TNF (Panel B). The presence of control IgG during TNF stimulation (Panel C) had no effect, while the presence of 3.3 µg/mL of cA2 during

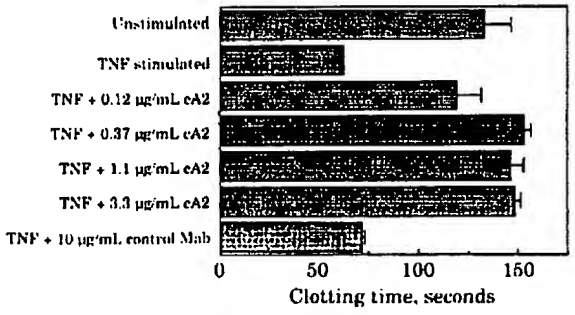


Figure 1. Effect of cA2 on the production of rhTNF-induced procoagulant activity by HUVE cells.

cA2 or control antibody were preincubated 30 min at room temperature with 25 ng/mL rhTNF prior to cell stimulation for 4 h at 37°C. Procoagulant activity was measured by determining the clotting time of recalcified human plasma after addition of the treated HUVE cell lysates. Data shown are the mean of duplicate wells ± SEM.

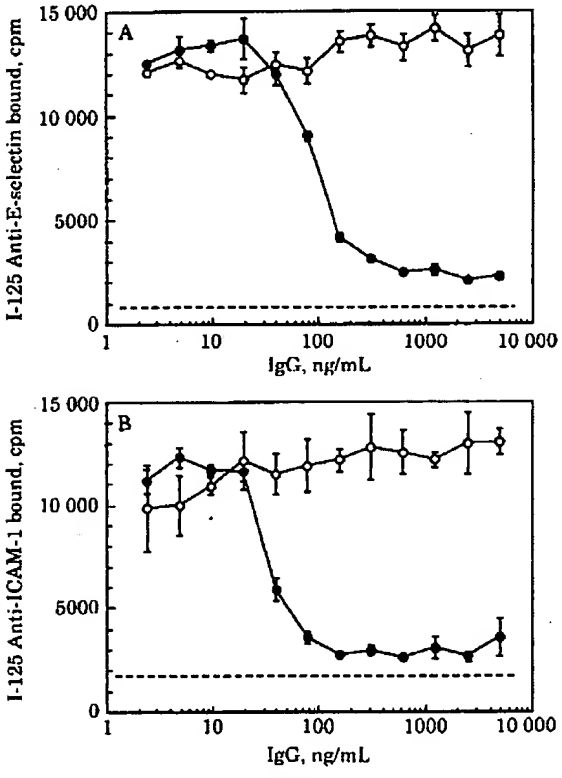


Figure 2. Neutralization of rhTNF-induced HUVE cell E-selectin and ICAM-1 expression by cA2.

Serial dilutions of cA2 (●) or control antibody (○) were mixed with 10 ng/mL of rhTNF and used to stimulate endothelial cell monolayers. E-selectin (Panel A) and ICAM-1 (Panel B) were detected after stimulation for 4 h and 23 h, respectively, using ^{125}I monoclonal antibodies specific for each adhesion protein. Data shown are the mean of triplicate wells ± SEM. The values obtained on unstimulated cells were 736 ± 65 cpm for E-selectin at 4 h and 1723 ± 149 cpm for ICAM-1 at 23 h (shown by dashed line).

CYTOKINE, Vol. 7, No. 1 (January 1995: 15–25)

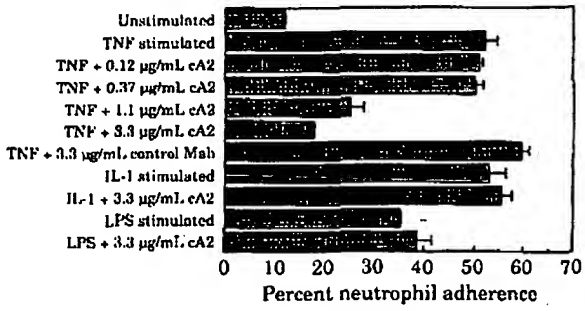


Figure 3. Neutralization of rhTNF-induced human neutrophil adhesion to HUVE cells by cA2.

HUVE cell monolayers (about 3×10^5 cells per well) were stimulated for 4 h with rhTNF (250 ng/mL), IL-1α (40 units/mL), J5 LPS (10 ng/mL) or medium alone (unstimulated). Incubations were performed in the presence or absence of the indicated concentrations of cA2 or control antibody. After washing, the stimulated HUVE cells were incubated an additional 45 min with human neutrophils (1×10^6 per well), and the number of neutrophils bound to the HUVE cells was determined by the myeloperoxidase assay. The data shown represents the average of triplicate wells ± SEM.

TNF stimulation (Panel D) markedly reduced the number of adherent neutrophils.

Effect of cA2 on TNF priming of human neutrophils

In vitro, TNF primes human neutrophils to produce superoxide upon subsequent stimulation with the chemotactic peptide f-met-leu-phe (FMLP).[32] The ability of cA2 to abrogate the TNF-priming phenomenon is shown in Figure 5. In the absence of cA2, TNF-primed (2 ng/mL) neutrophils produced as much as 40–45 nM superoxide upon stimulation with FMLP. TNF alone induced little or no superoxide production. cA2 was able to reduce the TNF-induced priming phenomenon in a dose-dependent manner, with levels as low as 1 μg/ml able to reduce superoxide production to the level typically seen with unprimed FMLP-stimulated neutrophils (7.85 nM in the experiment shown). Incubation of up to 100 μg/mL of control antibody with TNF had no effect on the subsequent production of superoxide upon FMLP stimulation (data not shown).

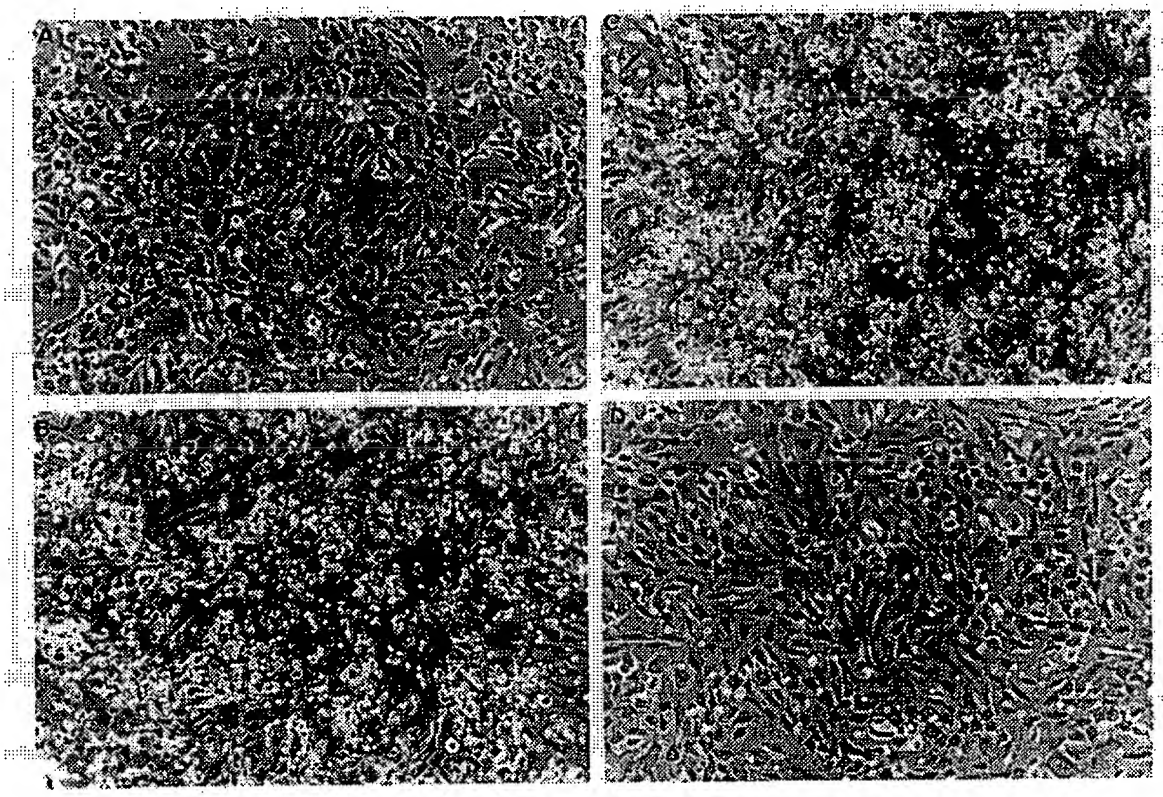


Figure 4. Phase contrast micrographs demonstrating neutrophil adherence to HUVE cells stimulated with rhTNF in the presence and absence of cA2.

HUVE cells were stimulated for 4 h with medium alone (A), 250 ng/mL rhTNF (B), 250 ng/mL rhTNF and 3.3 μg/mL control antibody (C) or 250 ng/mL rhTNF and 10 μg/mL cA2 (D). After stimulation, the ability of human neutrophils to adhere to the HUVE cell monolayer was assessed. Phase contrast micrographs were taken at 100-fold magnification.

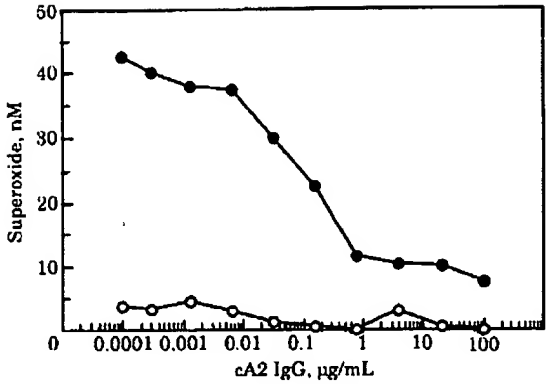


Figure 5. Effect of cA2 on the ability of rhTNF to prime human neutrophils.

Neutrophils (2.5×10^6 cells per mL) were primed with 2 ng/mL rhTNF in the presence of cA2 for 60 min, then the neutrophil activator FMLP (0.1 μM) (●) or medium (○) was added and the incubation continued for 10 min. Superoxide ion production was detected via the oxidation of cytochrome C. The data represent the average of duplicate wells with the background signal observed in parallel samples containing superoxide dismutase subtracted. Superoxide ion production by unprimed neutrophils exposed to FMLP was 7.85 nM.

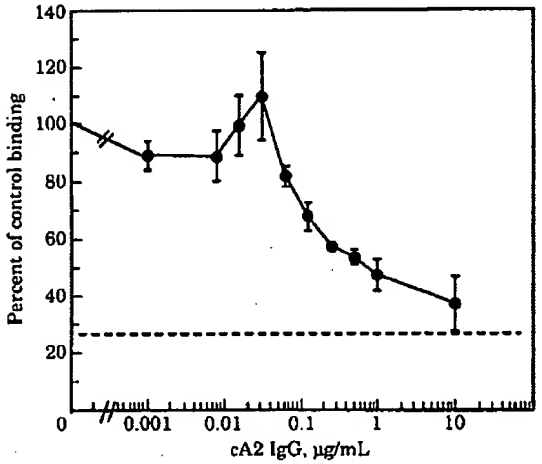


Figure 6. Binding of ^{125}I-rhTNF to U937 cell membranes in the presence of cA2.

U937 membranes were incubated for 3 h at 0°C with ^{125}I-rhTNF (45 pM) and the indicated concentration of cA2. ^{125}I-rhTNF bound to membranes was then separated by filtration and counted. Data are expressed as the mean ± SEM (three experiments) percent of binding in the absence of cA2 (1100 cpm). Binding of ^{125}I-rhTNF in the presence of 10 μg/mL negative control antibody was 96 ± 1%. Binding of ^{125}I-rhTNF in the presence of 40 nM unlabelled TNF (nonspecific background) was 26 ± 9% (shown by dashed line).

Effect of cA2 on TNF binding to receptor

The inhibition of TNF biological activity by cA2 presumably occurs as a result of the ability of cA2 to bind to soluble TNF, thereby inhibiting its interaction with cellular receptors. In order to directly demonstrate this in vitro, experiments were performed using both a commercially-available TNF receptor binding assay and recombinant immunoadhesion constructs of the human p55 and p75 TNF receptors. Figure 6 shows that cA2 inhibits the binding of radiolabelled TNF to a preparation of U937 monocytic cell membranes which contain TNF receptors. U937 cells have been previously shown to express both the p55 and p75 TNF receptors.[33] cA2 inhibition was dose-dependent, with 50% inhibition observed at 0.1 μg/mL.

Similarly, the binding of radiolabelled TNF to recombinant constructs containing the extracellular domain of either the p55 or p75 TNF receptor was inhibited by cA2 (Fig. 7). Inhibition by cA2 was dose-dependent, and 50% inhibition of binding to either receptor construct was evident at about 0.03 μg/mL cA2.

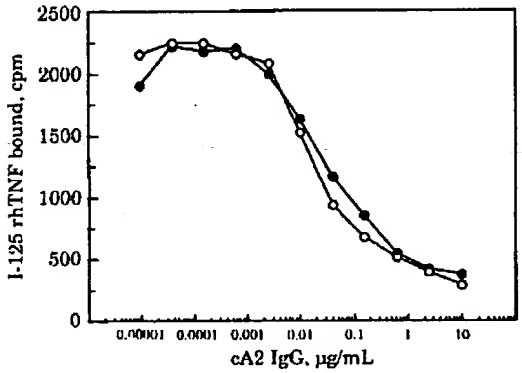


Figure 7. Binding of ^{125}I-rhTNF to p55 or p75 receptor fusion protein in the presence of cA2.

Serial dilutions of cA2 in 4 ng/mL ^{125}I-rhTNF were incubated on p55 (●) or p75 (○) receptor-coated microtiter wells for 1 h at 37°C. The data represent the mean of duplicate wells. Binding of ^{125}I-rhTNF in the presence of 10 μg/mL negative control antibody was 2270 cpm.

Effect of cA2 in a transgenic mouse model

Transgenic mice have been generated in which human TNF is constitutively expressed by their T cells.[25] These animals show elevated human TNF serum levels (0.01–7 units/mL) and develop a lethal wasting syndrome resulting in 80%–100% mortality at 10–18 weeks after birth. The ability of cA2 to prevent mortality in the transgenic mouse line Tg211 is shown in the survival curves of Figure 8. There was complete survival (15/15) to the 8 week endpoint in 3 week old animals administered cA2 twice-weekly at doses of 8 or 2 mg/kg. In the group administered the lowest dose of cA2, 0.5 mg/kg, there was a final 93% survival rate (14/15). By contrast,

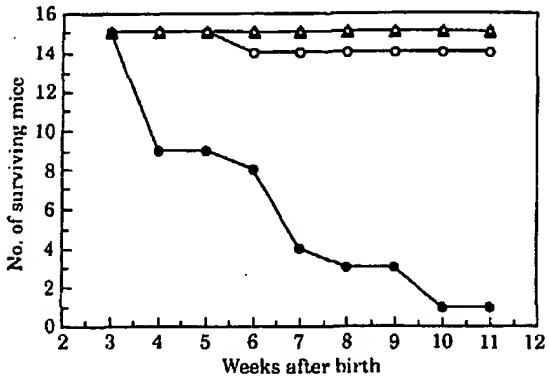


Figure 8. Survival curves of rhTNF transgenic mice treated with 0.5 (○), 2 (▲) and 8 (□) mg/kg cA2 IgG or 8 mg/kg control antibody (●).

Treatments were given twice weekly beginning at 3 weeks of age and continued for 8 weeks.

the control antibody group had a final survival rate of 7% (1/15). The difference between the least protective cA2 group (0.5 mg/kg) and the control group was highly statistically significant, log-rank $P = 0.0001$. The beneficial effect of cA2 was also evident when comparing weight gain between groups. Table 3 shows the average weight for each group at study entry (week 3), at study mid-point (week 7), and at study termination (week 11). Animals in the three cA2 treatment groups showed significant weight gain throughout the 8-week study which averaged 134%. By contrast, the control group showed only a modest (49%) weight gain throughout the 8-week study. Even this modest weight gain in the control group may be an overestimation since the control animals that died during the study may have experienced the least weight gain; however, they were obviously excluded from the later time points.

DISCUSSION

In this study we evaluated the effect of the chimeric anti-human TNF monoclonal antibody cA2 on both the in vitro and in vivo biological activities of TNF. cA2 exhibited potent neutralizing activity against TNF-mediated effects on human fibroblast, neutrophil and endothelial cell cultures. In the bioassays examined, 4 μg/mL of cA2 was sufficient to prevent the biological effects of an optimal challenge dose of TNF. The effects of cA2 were dose-dependent and highly specific. Incubation of cA2 with TNF directly inhibited the binding of TNF to receptor-containing cell membrane preparations and to recombinant constructs of the human p55 and p75 TNF cellular receptors. The neutralizing activity of cA2 could also be demonstrated in vivo, as evidenced by the ability of cA2 to reverse both the phenotype and accelerated mortality seen in transgenic mice that express the human TNF gene. In this case, cA2 efficacy was demonstrated in a chronic disease model by administering the drug over a period of 8 weeks. The potency of cA2 was evidenced by the suppression of mortality and weight loss with cA2 doses as low as 0.5 mg/kg administered twice weekly. These observations support and extend our previous studies which showed cA2 to be a high-affinity, highly-specific, TNF neutralizing antibody.[22]

The data presented for cA2 are consistent with the previously reported benefit of anti-TNF therapy in animal models of bacterial sepsis. Sepsis is a complex syndrome and it may be necessary to develop therapeutic modalities which target multiple disease mediators. Although clinical trials using passive anti-TNF immunotherapy are underway,[34] no efficacy data have been reported.

How do these findings relate to the potential application of cA2 to human autoimmune disease? In the case of RA, TNF has been implicated as a primary mediator of the chronic inflammation, although other

TABLE 3. Mortality and weight changes in TNF transgenic mice treated with cA2

Group	Study entry (week 3)	Study midpoint (week 7)		Study termination (week 11)	
	Average wt (g)	Number of Survivors	Average wt (g)	Number of Survivors	Average wt (g)
8 mg/kg cA2	7.4	15	16.0	15	20.0
2 mg/kg cA2	9.3	15	16.9	15	19.6
0.5 mg/kg cA2	7.6	14	15.9	14	16.8
8 mg/kg control antibody	10.0	4	11.4	1	14.9

Three week old Tg211 transgenic mice expressing rhTNF (n = 15 per group) were injected twice weekly with cA2 or an isotype-matched control antibody. Weights were measured weekly and represent the average of surviving animals.

inflammatory cytokines are also important.[16, 35] TNF has been shown in vitro to cause proliferation of synovial cells[36] as well as fibroblasts[20] which could directly contribute to pannus formation and fibrosis, respectively. This correlation is strengthened by the report that TNF is produced by cells at the cartilage-pannus junction[37] and is secreted spontaneously by cultured cells from synovial fluid from RA patients.[19] Thus, the ability of cA2 to block TNF-induced mitogenesis of human diploid fibroblasts, as well as their secretion of IL-6, may be relevant to this phenomenon. Pannus formation in diseased joints is accompanied by increased expression of ICAM-1,[14] which can be upregulated by several cytokines including TNF and IL-1, and leads to the infiltration of mononuclear cells. In the present study, cA2 has been shown to block TNF-induced ICAM-1 (as well as E-selectin) expression by cultured human endothelial cells. The blocking by cA2 of ICAM-1 and E-selectin expression directly resulted in decreased adherence of human neutrophils to endothelial cell monolayers. Neutrophils are numerous in synovial fluid from RA patients and it is likely that their activation leads to further necrosis of tissue. It may therefore also be important that cA2 can block the priming of neutrophils by TNF. Although not investigated in this study, TNF also is involved in the induction of collagenase in synovial cells,[38] the inhibition of prostaglandin synthesis by articular chondrocytes[39] and the stimulation of bone resorption.[40] Moreover, it has been shown that the use of an anti-human TNF monoclonal antibody completely neutralizes development of disease in a human TNF transgenic model of arthritis.[20] The potential for reducing RA joint disease using an anti-TNF antibody approach has also been demonstrated in a murine model of collagen-induced arthritis.[41] Mice were treated weekly with 10 mg/kg of anti-murine TNF antibody, with treatment beginning either before immunization with type II collagen or after immunization and onset of clinical arthritis. In both circumstances, the anti-TNF antibody reduced swelling of the paws and severity of disease by histopathological assessment of the arthritic joints.

While numerous cytokines such as TNF, IL-1, IL-6, GM-CSF and TGF-β have been identified in joint synovial fluids of patients with RA,[15] recent studies by Feldmann and colleagues suggest that TNF may regulate the levels of these other cytokines.[42, 43] Earlier studies have demonstrated that TNF is a potent inducer of IL-1 in endothelial cells[44] and monocytes,[45] as well as of IL-6[46] and GM-CSF.[47] Cells cultured from the synovial fluid from the diseased joints of RA patients continue to spontaneously produce bioactive IL-1. The addition of a polyclonal, neutralizing anti-human TNF antibody specifically reduced the levels of bioactive IL-1β produced.[44] In a similar set of experiments, levels of bioactive GM-CSF were shown to be significantly reduced by the addition of neutralizing anti-human TNF antibodies to the media of cultured synoviocytes from RA patients.[45] These results provide a clear rationale for the evaluation of anti-TNF therapy in RA, and results from initial studies to assess safety and efficacy in RA patients are encouraging.[24]

TNF also has been cited as a potential mediator in several other autoimmune diseases. Elevated serum levels of TNF correlate with relapsing ulcerative colitis and chronic Crohn's disease[48] and TNF in the stool of patients with inflammatory bowel disease may be a marker which correlates with disease activity.[49] A temporary remission has been described in a Crohn's patient treated with cA2.[23] In this case report, a Crohn's patient who was unresponsive to conventional treatment received two doses of cA2 (10 mg/kg) spaced two weeks apart. Over a period of ten weeks after treatment, the patient gained weight and showed reduction in standard indexes of disease activity as well as complete endoscopic remission. Symptoms returned approximately 3 months after treatment. Anti-TNF antibodies have also been evaluated in experimental allergic encephalomyelitis (EAE), an autoimmune demyelinating disease in mice which mimics multiple sclerosis. Treatment of mice with neutralizing anti-TNF antibodies prevented transfer of EAE symptoms by a T cell clone[50] and delayed relapse caused by bacterial superantigen.[51]

It should be pointed out that the precise molecular role(s) of TNF in each of these disease states remains to be elucidated, and that further understanding of localized versus systemic effects of this cytokine is critical to the rational design of targeted anti-TNF therapy. This is particularly important as the role of TNF may be beneficial in certain disease states and under certain conditions.[52] Highly specific, potent neutralizing agents such as cA2 provide a valuable tool for the elucidation of TNF biology in human disease, and may serve as new forms of therapeutic intervention in those cases where a causal relationship between aberrant TNF expression and disease pathology can be established. The results of the present study demonstrate the potential usefulness of cA2 and provide a rationale for its continued evaluation in human disease.

MATERIALS AND METHODS

Reagents

The monoclonal chimeric mouse/human anti-TNF IgG_1 (cA2) antibody was isolated from concentrated hybridoma cell supernatant by Protein A-Sepharose chromatography and ion exchange chromatography. Chimeric mouse/human 7E3 anti-platelet IgG_1, chimeric mouse/human 17-1A anti-tumour antigen IgG_1, and chimeric mouse/human MT-412 anti-CD4 IgG_1 were also purified by Protein A-Sepharose

chromatography and used as isotype-matched, irrelevant antibody controls. Lyophilized, carrier free recombinant human TNF (rhTNF) was obtained from Dainippon, Osaka, Japan; from Suntory, Osaka, Japan; and from Biosource, Camarillo, CA. The anti-E-selectin antibody H18/7 was a gift from Dr M. Bevilacqua, formerly at Brigham and Women's Hospital, Boston, MA. Anti-ICAM-1 antibody #11 was a gift from Dr G. Riethmuller, University of Munich, Germany. H18/7 and #11 antibodies were iodinated to a specific activity of 2–3 μCi/μg using Iodobeads (Pierce, Rockford, IL) as per the manufacturer's instructions. Human umbilical vein endothelial (HUVE) cells were obtained from Cell Systems, Kirkland, WA. The FS-4 fibroblast line was established and has been maintained at the New York University Medical Center. Recombinant constructs of the p55 and p75 human TNF receptors were a gift from Dr Bernard Scallon, Department of Molecular Biology, Centocor, Inc. Briefly, the p55-sf2 construct contains the extracellular domain of human p55 fused to eight amino acids of human antibody J sequence followed by all three constants domains of human IgG1. It is disulfide bonded to a human kappa light chain constant region. The p75P-sf2 construct is the same as p55-sf2 except it contains a truncated form of the extracellular domain of human p75. Both constructs were purified from cell culture supernatant by Protein A affinity chromatography.

Cell culture

Human umbilical vein endothelial (HUVE) cells were grown in complete HUVE medium (Cell Systems, Kirkland, WA) containing 15% fetal bovine serum (FBS; Hyclone, Logan UT) supplemented with growth factor (CS-HBGF-I/H; Cell Systems, Kirkland, WA) on T-75 tissue culture flasks (Corning, Corning, NY). All plasticware used for HUVE cell culture was coated first with attachment factor (Cell Systems, Kirkland, WA) by wetting the surface with attachment factor and removing the excess fluid prior to introducing cells. Cells were serially passaged by splitting 1:3 in the same medium every 3–5 days and all assays were performed on cells at passages 2–4.

FS-4 fibroblasts were grown in modified Eagle's medium (MEM) containing 5% FBS in T-75 flasks. Cells were serially passaged by splitting 1:5 in the same medium every 2 weeks; cells at passage level 13 to 15 were used in the experiments.

Mitogenesis assay

FS-4 fibroblasts were seeded at 8×10^3 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured for 18 h at 37°C. Recombinant human TNF was diluted in complete MEM to final concentrations of 0, 0.1, 0.5 and 2 ng/mL in the absence or presence of 4 μg/mL test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to quintuplicate wells and incubated at 37°C for 5 days. The cells were rinsed with phosphate buffered saline (PBS) pH 7.2, then fixed by adding 50 μL/well of 10% formalin in PBS for 15 min at room temperature. The fixed cells were then stained with 50 μL/well of 0.05% naphthol blue black in 9% acetic acid, 0.1 M sodium acetate for 30 min at room temperature. The cells were then rinsed thoroughly with distilled water, and the bound dye was eluted with 150 μL/well of 50 mM NaOH. Absorbance of the eluted dye was determined at 630 nm.

Assay for IL-6

FS-4 fibroblasts were seeded at 2×10^4 cells/well in a 96-well tissue culture plate in MEM containing 5% FBS and cultured overnight at 37°C. Recombinant human TNF was diluted to final concentrations of 0, 0.3, 1.5 and 7.5 ng/mL in the absence or presence of 4 μg/mL test antibody. The samples were preincubated for 20 min at room temperature, then 0.1 mL was added to duplicate culture wells and the incubation continued for 18 h. The cell supernatants from duplicate wells were pooled and stored at -20°C. The amount of IL-6 present in each sample was determined using an ELISA-based plate assay (Quantikine IL-6, R&D Systems, Minneapolis, MN) as described by the manufacturer.

Procoagulant activity assay (PCA)

The PCA assay was performed on HUVE cells plated at 1.5×10^5 cells/well in 24-well tissue culture plates (Falcon) coated with attachment factor. Confluent monolayers were washed three times with 500 μL/well of RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 1% FBS and 0.3 mg/mL L-glutamine (JRH Biosciences, Lenexa, KS). Antibody samples were tested at 3.3, 1.1, 0.37 and 0.12 μg/mL for neutralization of rhTNF at a final concentration of 25 ng/mL. Medium alone (no rhTNF) and medium plus 10 μg/mL cA2, were used as negative controls while medium containing rhTNF at 25 ng/mL (no antibody) was used as a positive control. All dilutions were prepared in complete RPMI medium and were preincubated for 30 min at room temperature prior to incubation with the endothelial cells. Test solutions were dispensed into duplicate wells, 500 μL/well, and incubated for 4 h at 37°C. The test solutions were removed with gentle aspiration and the cells were washed as described previously. Three hundred microliters of complete RPMI were dispensed into each well and the plates were immediately frozen at -70°C. Cell lysates were prepared by thawing the plates at room temperature, resuspending all cell debris, and freezing and thawing each plate two more times. The plasma clotting assay was performed after equilibrating all reagents at 37°C. Clotting was initiated by mixing 0.1 mL of fresh, citrated human plasma, 0.1 mL of cell lysate and 0.1 mL of 30 mM $CaCl_2$ in a glass tube and incubating at 37°C. The time required for the clot to form (by visual inspection) was recorded and the mean and standard deviation from duplicate cell lysates were calculated.

Adhesion protein assays

The E-selectin and ICAM-1 assays were performed on HUVE cells plated at 5×10^4 cells/well in 96-well tissue culture plates (Costar 3596, Cambridge, MA) coated with attachment factor. Confluent monolayers were gently washed twice using a multi-channel pipettor with 150 μL/well of HUVE medium. Twofold serial dilutions of cA2 or c17-1A were prepared in medium containing 10 ng/mL rhTNF. Medium alone was used as a negative control while medium containing 10 ng/mL rhTNF was used as a positive control. Test solutions were dispensed into triplicate wells, 100 μL/

well, and incubated for 4 h (E-selectin assay) or 23 h (ICAM-1 assay) at 37°C. The test solutions were removed with a multichannel pipettor and the iodinated probes were added. Expression of adhesion protein was detected by incubating the cells for 2 h at room temperature with ^{125}I anti-E-selectin antibody H18/7 F(ab')$_2$ or ^{125}I-anti-ICAM-1 #11 IgG diluted to 10 μg/mL in complete HUVE medium (300000 cpm/100 μL/well). The cells were then washed 4× with RPMI-1640 containing 10% FBS, and the well contents solubilized and counted for ^{125}I in a gamma counter.

Neutrophil adhesion and priming assays

Neutrophils were isolated from 100 mL of fresh human blood drawn into heparin. Ten millilitres of blood were layered onto 5 mL of Mono-poly resolving medium (Flow Labs, McLean, VA) in a 15 mL conical centrifuge tube and centrifuged at 750 × *g* for 30 min at room temperature. Additional centrifugation for 30 min at 900 × *g* was generally required to completely separate the bands of cells. The top band containing T and B cells was discarded and the lower band containing polymorphonuclear cells was collected. The neutrophils were washed with Hank's buffered saline (without magnesium and calcium) and were resuspended in RPMI-1640/10% FBS.

For the adhesion assay, HUVE cells grown in 24-well tissue culture plates were stimulated for 4 h at 37°C with rhTNF (250 ng/mL), IL-1α (40 units/mL; Genzyme, Boston, MA) or *E. coli* J5 LPS (10 ng/mL; List Biological Laboratories, Campbell, CA) in RPMI-1640/10% FBS containing the indicated concentration of test antibody, or with medium alone. The HUVE monolayers, containing about 3 × 10^5 cells per well, were then washed once and overlaid with 0.1 mL of RPMI-1640/10% FBS containing 1 × 10^6 neutrophils. After incubation for 45 min at 37°C, the monolayers were gently washed three times with RPMI-1640/10% FBS and then solubilized with 0.25 mL of 50 mM potassium phosphate pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The solubilized monolayer samples were then quantitated using the myeloperoxidase assay in which known quantities of isolated neutrophils were processed in the same manner and used to generate a standard curve. Standard samples were analysed in duplicate while test samples were tested in triplicate. Reagents were added to the wells of a 96-well microtiter plate in the following order: 55 μL of 80 mM potassium phosphate pH 5.4; 15 μL of sample, standard or blank; 20 μL of 0.3 mM hydrogen peroxide in 80 mM potassium phosphate pH 5.4; 10 μL of 16 mM 3,3',5,5'-tetramethylbenzidine in N, N'-dimethylformamide. The plate was incubated for 15 min at room temperature and the reaction stopped by adding 100 μL of 1 M phosphoric acid per well. The optical density of each well was read at 450 nm. Photomicroscopy of HUVE cell monolayers at 100× magnification was performed using a phase contrast Nikon IMT-2 inverted research microscope.

For neutrophil oxidative burst assays, 2.5 × 10^6 cells/mL were primed with 2.0 ng/mL rhTNF in the presence of the indicated concentration of test antibody for 60 min at 37°C. Primed cells were activated (or mock-activated) with 0.1 μM FMLP (Sigma, St. Louis, MO) for 10 min at 37°C in the presence of 1 mg/mL cytochrome C (Sigma, St. Louis, MO). The cells were then microcentrifuged for 5 min and the OD of the supernatants read at 550 nm. Duplicate samples containing 10 μg/mL superoxide dismutase (SOD; Sigma) were run in parallel and the background OD obtained subtracted from samples without SOD. The results were converted to nM superoxide ion using the extinction coefficient for (reduced cytochrome c) – (oxidized cytochrome c) for a 3 mm path length.[53]

Receptor binding assays

^{125}I-rhTNF binding to U937 membranes was performed using a New England Nuclear (Boston, MA) ligand binding kit. Briefly, 45 pM of ^{125}I-rhTNF (40–50 μCi/μg), the indicated concentrations of test antibody and U937 membranes provided, were incubated as described in the manufacturer's instructions in a final volume of 250 μl for 3 h on ice. Membrane bound tracer was separated from free tracer by vacuum filtration over GF/C filters. The filters were washed 2 × 4 ml with the wash buffer provided. Data were expressed as the mean ± SEM of three separate experiments and graphed as percent of control ^{125}I-rhTNF binding in the absence of antibody (approximately 1100 cpm). Binding of tracer in the presence of 10 μg/ml of a negative control antibody was 96% ± 1%.

To assess ^{125}I-rhTNF binding to recombinant constructs of the p55 and p75 cellular receptors for TNF, 50 μL of a 5 μg/mL solution of either p55 or p75 receptor fusion proteins in PBS was incubated on polystyrene 96-well plates for 1 h at 37°C. The wells were washed and blocked for 1 h at 37°C with PBS containing 1% BSA. Equal volumes (25 μL each) of serially diluted cA2 in PBS/1% BSA and 2 × ^{125}I-rhTNF (final concentration = 4 ng/mL) were added to duplicate wells. Plates were incubated for 1 h at 37°C, washed 2 × 200 μL with PBS and the radioactivity bound was counted in a gamma counter. Binding of tracer in the presence of 10 μg/mL negative control antibody was 2270 cpm.

Transgenic mouse protection model

Tg211 transgenic mice were bred as previously described[19] and randomly divided into groups of 15 animals each. These groups received twice-weekly intraperitoneal injections of 10 μL per gram average body weight to achieve a final dose of 0.5, 2 and 8 mg/kg cA2 IgG. A fourth group received 8 mg/kg of an isotype-matched control antibody. Investigators (LP and GK) performing the experiment at the Hellenic Pasteur Institute were blinded with respect to the drug each treatment group received during the course of the study. Injections of test antibody were initiated when the mice reached 3 weeks of age, and the study was terminated after 8 weeks of treatment. Average weight and mortality in each group was recorded weekly.

Acknowledgements

The authors wish to thank Drs Bevilacqua, Riethmuller and Scallon for providing reagents necessary to complete this study. The authors also thank E. Wilson and T. Ely for technical assistance and J. Wendel for preparation of the manuscript.

REFERENCES

1. Beutler B, Cerami A (1988) Tumor necrosis, cachexia, shock and inflammation: a common mediator. Ann Rev Biochem 57:505-18.

2. Vilcek J, Lee TH (1991) Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem 266:7313-7316.

3. Balkwill FR, Burke F (1989) The cytokine network. Immunol Today 10:299-304.

4. Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino Jr MA, Cerami A, Shires GT, Lowry SF (1988) Cytokine appearance in human endotoxemia and primate bacteremia. Surg Gynecol Obstet 166: 147–153.

5. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey III TJ, Zentella A, Albert JD, Shires GT, Cerami A (1986) Shock and tissue injury induced by recombinant human cachectin. Science 234:470-473.

6. Tracey KJ, Lowry SF, Fahey III TJ, Albert JD, Fong Y, Hesse D, Beutler B, Manogue KR, Calvano S, Wei H, Cerami A, Shires GT (1987) Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. Surg Gynecol Obstet 164:415-422.

7. Beutler B, Milsark IW, Cerami AC (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229:869-871.

8. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature 330:662-664.

9. Hinshaw LB, Tekamp-Olson P, Chang ACK, Lee PA, Taylor Jr FB, Murray CK, Peer GT, Emerson Jr TE, Passey RB, Kuo GC (1990) Survival of primates in LD_{100} septic shock following therapy with antibody to tumor necrosis factor (TNFα). Circ Shock 30:279-292.

10. Fiers W (1991) Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS Letters 285(2):199-212.

11. Dinarello CA (1992) Interleukin-1 and tumor necrosis factor: effector cytokines in autoimmune diseases. Semin Immunol 4(3):133-45.

12. Jacob CO (1992) Studies on the role of tumor necrosis factor in murine and human autoimmunity. J Autoimmun 5 Suppl A:133-43.

13. Harris ED Jr (1990) Rheumatoid arthritis. Pathophysiology and implications for therapy. N Engl J Med 322:1277-1289.

14. Sewell KL, Trentham DE (1993) Pathogenesis of rheumatoid arthritis. Lancet 341:283-286.

15. Brennan FM, Maini RN and Feldmann M (1992) TNFα—A pivotal role in rheumatoid arthritis? Br J Rheumatol 31:293-98.

16. Kollias G (1993) Tumor necrosis factor: A specific trigger in arthritis. In: 51st Forum in Immunology, TNF in Pathology: old facts and new questions, Research in Immunology, 5:342-347.

17. DiGiovine F, Nuki G, Duff G (1988) Tumor necrosis factor in synovial exudates. Ann Rheum Dis 47:768-772.

18. Saxne T, Palladino MA Jr, Heinegard D, Talal N, Wollheim FA (1988) Detection of tumor necrosis factor α but not tumor necrosis factor β in rheumatoid arthritis synovial fluid and serum. Arthr Rheum 31:1041-1045.

19. Brennan FM, Chantry D, Jackson AM, Maini RN, Feldmann M (1989) Cytokine production in culture by cells isolated from the synovial membrane. J Autoimmun 2 S:177-186.

20. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, Kollias G (1991) Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. EMBO J 10:4025-4031.

21. Brennan FM, Field M, Chu CQ, Feldmann M, Maini RN (1991) Cytokine expression in rheumatoid arthritis. Br J Rheumatol 30 S1:76-80.

22. Knight DM, Trinh H, Le J, Siegel S, Shealy D, McDonough M, Scallon B, Moore MA, Vilcek J, Daddona P, Ghrayeb J (1993) Construction and initial characterization of a mouse/human chimeric anti-TNF antibody. Mol Immunol 30:1443-53.

23. Derkx B, Taminiau J, Randema S, Stronkhorst A, Wortel C, Tytgat G, van Deventer S (1993) Tumour-necrosis-factor antibody treatment in Crohn's disease. Lancet 342:173-174.

24. Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Katsikis P, Brennan FM, Walker J, Bijl H, Ghrayeb J, Woody JN (1993) Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor-α. Arthritis Rheum 36:1681-1690.

25. Probert L, Keffer J, Corbella P, Cazlaris H, Patsavoudi E, Stephens S, Kaslaris E, Kioussis D, Kollias G (1993) Wasting, ischemia and lymphoid abnormalities in mice expressing T cell-targeted human tumor necrosis factor transgenes. J Immunol 151:1894-1906.

26. Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M (1986) Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med 163:632-643.

27. Kohase M, Henriksen-DeStefano D, May LT, Vilcek J, Sehgal PB (1986) Induction of β_2-Interferon by Tumor Necrosis Factor. A homeostatic mechanism in the control of cell proliferation. Cell 45:659-666.

28. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr (1986) Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin-1. Proc Natl Acad Sci USA 83:4533-4537.

29. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B (1989) Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 243:1160-1165.

30. Pober JS, Lapierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA Jr (1987) Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin-1 species. J Immunol 138:3319-3324.

31. Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA (1985) Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 82:8667-8671.

32. Berkow RL, Wang D, Larrick JW, Dodson RW, Howard TH (1987) Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. J Immunol 139:3783-3791.

33. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W (1993) Tumor necrosis factor α (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med 177:1277-1286.

34. Pennington JE (1993) Therapy with antibody to tumor necrosis factor in sepsis. Clin Infect Dis 17 (Suppl 2):S515-S519.

35. Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Abney E, Buchan G, Barrett K, Barkley D, Chu A, Field M, Maini RN (1990) Cytokine production in the rheumatoid joint: implications for treatment. Ann Rheum Dis 49:480-486.

36. Gitter BD, Labus JM, Lees SL, Scheetz ME (1989) Characteristics of human synovial fibroblast activation by IL-1β and TNFα. Immunology 66:196-200.

37. Chu CQ, Field M, Feldmann M, Maini RN (1991) Localization of tumor necrosis factor α in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 34:1125-1132.

38. Dayer JM, Beutler B, Cerami A (1985) Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med 162:2163-2168.

39. Saklatvala J (1986) Tumor necrosis factor α stimulates resorption and inhibits synthesis or proteoglycan in cartilage. Nature 322:547-549.

40. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR (1986) Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 319:516-518.

41. Williams RO, Feldmann M, Maini RN (1992) Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 89:9784-9788.

42. Brennan FM, Chantry D, Jackson A, Maini RN, Feldmann M (1989) Inhibitory effect of TNFα antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet ii:244-247.

43. Haworth C, Brennan FM, Chantry D, Turner M, Maini RN, Feldmann M (1991) Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: regulation by tumor necrosis factor-α. Eur J Immunol 21:2575-2579.

44. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D (1986) Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin-1. J Exp Med 163:1363-1375.

45. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, O'Connor JV (1986) Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. J Exp Med 163:1433-1450.

46. Van Damme J, Opdenakker G, Simpson RJ, Rubira MR, Cayphas S, Vink A, Billiau A, Van Snick J (1987) Identification of the human 26-kD protein, interferon β_2 (IFN-β_2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin-1 and tumor necrosis factor. J Exp Med 165:914-919.

47. Seelentag WK, Mermod J-J, Montesano R, Vassalli P (1987) Additive effects of interleukin-1 and tumor necrosis factor-α on the accumulation of the three granulocyte and macrophage colon-stimulating factor mRNAs in human endothelial cells. EMBO J 6:2261-2265.

48. Murch SH, Lamkin VA, Savage MO, Walker Smith JA, MacDonald TT (1991) Serum concentrations of tumour necrosis factor-α in childhood chronic inflammatory bowel disease. Gut 32:913-917.

49. Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT (1992) Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. Lancet 339:89-91.

50. Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, Clark RB (1990) An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. J Exp Med 172:1193-1200.

51. Brocke S, Gaur A, Piercy C, Gautam A, Gijbels K, Fathman CG, Steinman L (1993) Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. Nature 365:642-644.

52. Bagby GJ, Plessala KJ, Wilson LA, Thompson JJ, Nelson S (1991) Divergent efficacy of antibody to tumor necrosis factor-α in intravascular and peritonitis models of sepsis. J Inf Dis 163:83-88.

53. Metcalf JA, Gallin JI, Nauseef WM, Root RK (1986) Laboratory Manual of Neutrophil Function, Raven Press, New York.

BC685.C5 T5
Mic.
Adams
MC

STIC-ILL

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

Pergamon

Thrombosis Research, Vol. 81, No. 3, pp. 315–326, 1996

0049-3848/96 $12.00 + .00

PII S0049-3848(96)00003-5

TNF-α SUPPRESSES IL-6 UPREGULATION OF PROTEIN S IN HepG-2 HEPATOMA CELLS

W. Craig Hooper, Donald J. Phillips, and Bruce L. Evatt
Hematologic Diseases Branch, Division of HIV/AIDS, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Ga 30333

(Received 21 July 1995 by Editor D.A Triplett; revised/accepted 14 December 1995)

Abstract The pathogenesis of disseminated intravascular coagulation (DIC) has, in part, been attributed to the impairment of the natural anticoagulant protein C/protein S pathway. DIC, which frequently occurs during sepsis, has been linked to cytokines that can induce or modulate procoagulant activity. Three of these cytokines, IL-1α, IL-6, and TNF-α have been reported to be increased in the early stages of sepsis. In the present study, we have stimulated HepG-2 hepatoma cell cultures with recombinant human IL-1α, IL-6, TNF-α, and oncostatin M (OSM). The results demonstrated that TNF-α, and to a lesser degree, IL-1α, could significantly suppress IL-6 upregulation of protein S, whereas the effects of OSM was only suppressed by the combination of IL-1α and TNF-α. The combination of IL-1α and TNF-α also suppressed protein S production below that of control or basal levels. These results indicate that IL-1α and TNF-α may play important regulatory roles in coagulation.

The protein C/protein S anticoagulant pathway is critical in maintaining a balance between activation and inactivation of the coagulation cascade. The clinical importance of this pathway has been demonstrated by the association of thrombosis with either congenital or acquired deficiency of either protein C or protein S (1-10). Several recent studies have

Key Words: IL-1, IL-6, TNF, Thrombosis, HepG-2, Protein S
Corresponding Author: W. Craig Hooper, Ph.D., MS-D02, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, Ga 30333

suggested that this anticoagulant pathway could act as a pathophysiological link between coagulation and septic shock (6,9,11-17). The clinical significance of these anticoagulant proteins has been further delineated in both animal models and human studies in which infusions of either protein C or protein S decreased the hypercoagulable complications associated with disseminated intravascular coagulation (DIC) in sepsis (9,11,14,15).

Protein C, a vitamin K-dependent protein and zymogen of a serine protease, is activated following the interaction of thrombin with its receptor, thrombomodulin (18). Once activated, protein C forms a complex with its cofactor protein S, also a vitamin K-dependent protein, and enzymatically inactivates the thrombogenic factors Va and VIIIa (19-22), thereby inhibiting coagulation. Protein S exists as two forms in the plasma, free and bound. Approximately 40% of protein S is physiologically active and free in the plasma while the remainder is inactive and bound in a 1:1 stoichiometric complex with the C4b-binding protein (C4BP) (23-25). Although the pathophysiological events that contribute to the impairment of the protein C/protein S system are poorly understood, proinflammatory cytokines are known to play a role in procoagulant activation (17, 26-31) as well as in the regulation of thrombomodulin (27,32,33). Two cytokines known to be increased in sepsis, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), are thought to play a central but somewhat diverse role by not only causing tissue damage (34) but also by inducing the acute-phase response (35-38). While TNF-α has been considered a principal mediator of both procoagulant activity (26,27) and sepsis, (28-31,39), recent data from experimental endotoxemia in baboons have suggested that IL-6 may also be an intermediary factor in the activation of coagulation (17).

We have previously shown that protein S and its negative regulator, C4BP were upregulated by both IL-6 and OSM in the human hepatoma cell line, HepG-2 (40). We now show that TNF-α significantly suppresses the IL-6, but not the OSM effect on protein S. This observation is indicative of a negative regulatory role for TNF-α in the IL-6 mediated synthesis and regulation of the hepatocyte derived anticoagulant protein S.

MATERIALS AND METHODS

Cell culture

The HepG-2 human hepatocellular carcinoma cell line was obtained from the American Type Culture Collection, Rockville, MD, USA and propagated as adherent monolayer cultures which were incubated in a 37°C, 5% CO_2, humidified atmosphere. Cells were grown in Eagles's minimal essential medium that contained penicillin-streptomycin (50 U/ml and 50 μg/ml respectively); nonessential amino acids and sodium pyruvate (1 mM in Earle's BSS), and 10% fetal bovine serum (Gibco BRL, Grand Island, NY[§]). Experiments were performed with cells between passage levels 12-20. Cells were plated at a density of 1.0 X 10^5 cells/cm^2 in 2.0-cm^2 24-well or 0.32-cm^2 96-well culture plates (Costar, Cambridge, MA). When monolayers were about confluent, 24- 48-hours, supernatants were removed by aspiration and adherent cells washed with phosphate buffered saline. Fresh media (0.25-1 ml), with or without added cytokine, was then added to each well (zero time). Culture

fluids were harvested at scheduled intervals for protein S antigen quantification by enzyme-linked immun sorbent assay (ELISA). Each experimental determination was performed with fluids from 4-8 replicate culture wells.

Statistical analysis

Data presented here represent measurements from 4-8 replicate culture supernatants and are expressed as mean and standard deviation of the mean. Probability (P) values were determined (Student's t-test) from measurements of replicate culture fluids, and P values of $< .05$ were considered statistically significant. Additionally, experimental determinations were repeated a minimum of three times.

Cytokines

Recombinant human IL-1α, IL-6 and its soluble receptor gp80, OSM, and TNF-α were obtained commercially (R & D Systems, Inc., Minneapolis, MN) and employed at or near physiological concentrations as indicated in the RESULTS section. The manufacturer had determined endotoxin levels to be <0.1 ng per 1 μg of cytokine.

Antibodies

A goat anti-TNF R1 antibody (AB225-PB) against the 55-kDa human TNF R1 receptor, which reportedly does not cross-react with human recombinant TNF BPII (75-kDa TNF receptor) and exhibits TNF agonist activity on the human cell line A549, was purchased from R & D Systems, Inc. To determine if this antibody demonstrated agonist activity on the HepG-2 cell line, the antibody (10 μg/ml) was substituted for TNF-α in some experiments, and protein S levels in culture fluids were measured by ELISA and compared with those in untreated and TNF-α treated cultures.

ELISA

The ELISA used to quantify protein S in cell culture fluids has been previously described (41). Briefly, goat anti-protein S antiserum (American Diagnostica, Greenwich, CT) fractionated on DEAE-Sephadex A50 (fall through after equilibration in 0.1M Tris-HCl pH 8.3, 0.05M NaCl) was used as capture antibody (1.5 μg in 100 μl Tris-HCl, 0.05 M, pH 9.0 per well). Rabbit anti-human protein S second antibody (Sigma, St. Louis, MO) was used at 1:2000 in phosphate buffered saline, 5% normal goat serum, and detected with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham) diluted 1:1500 in the same buffer. HRP activity was measured against o-phenylenediamine (Sigma) dissolved at 0.4 mg/ml in 0.05 M citrate-phosphate buffer, pH 5.0 containing H_2O_2 (0.00133%). Absorbance at 490 nm was determined with an EL312e Microplate Reader (Biotek, Winooski, VT). Data reduction used the KinetiCalc software package (Biotek). Purified protein S used for calibration and as a positive control was obtained from American Diagnostica and has been previously characterized (10,41).

RESULTS

IL-6 treatment of HepG-2 hepatoma cells resulted in protein S levels which were significantly ($P < .05$-$.001$) increased over basal levels (Fig. 1). TNF-α, at 1 ng/ml, partially suppressed protein S upregulation by IL-6 in the HepG-2 hepatoma cells and completely suppressed the response at concentrations of ≥ 10 ng/ml (Fig. 1).

It was earlier observed that IL-6 had a modest negative effect on HepG-2 hepatocyte cell proliferation (40). In the current series of experiments, TNF-α or the combination of IL-6/TNF-α had no significant effect on proliferation over that of IL-6 alone (data not shown).

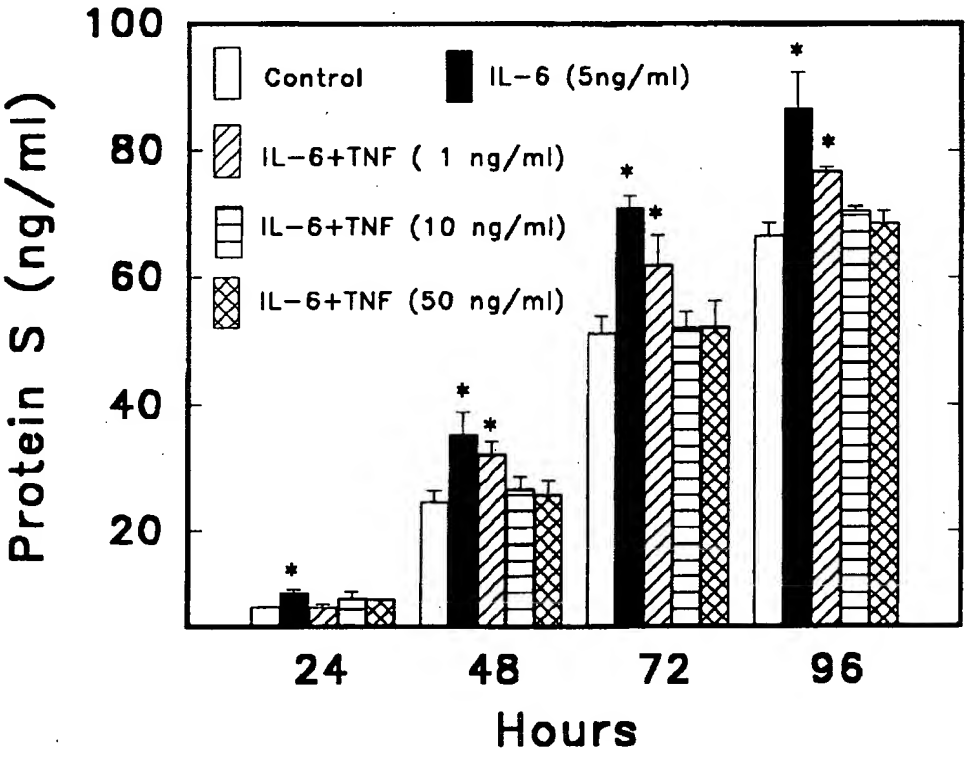


FIG. 1.

IL-6 upregulation of protein S in HepG-2 hepatoma cells was suppressed by TNF-α and was shown to be dose dependent. Error bars represent one standard deviation about the mean value.

The monoclonal antibody directed against the TNF 55 kDa receptor-I (anti-TNF RI) acted as an agonist by suppressing IL-6 mediated upregulation of protein S (Fig. 2). Although TNF-α and anti-TNF RI had similar suppressive effects, no synergism was bserved when both were used in combination (data not shown). The combination of IL-6 and its soluble receptor (sIL-6R), previously determined to result in a significant upregulation of protein S over that of IL-6 alone (40), was suppressed by both TNF-α (Fig.3) and anti-TNF RI (data not shown).

The effect of IL-1α was intermediate to that of TNF-α (Fig. 4 cf. Fig. 1). When IL-1α and TNF-α were combined, a synergistic effect was observed in which protein S levels were slightly below values seen in the control cells (Fig. 4).

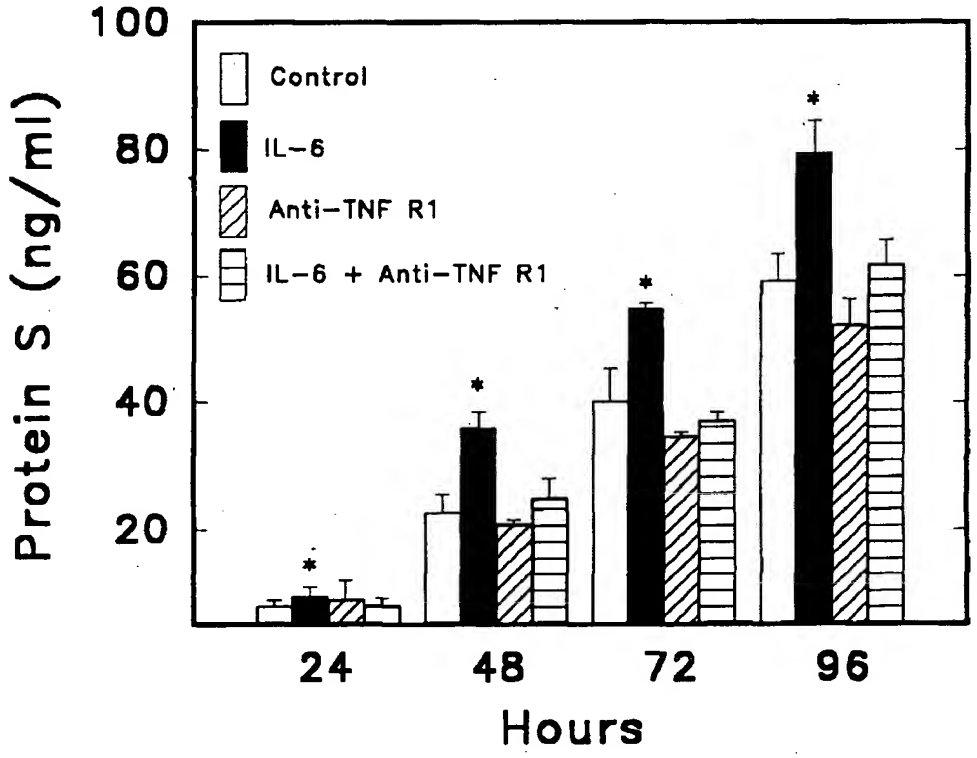


FIG. 2.

IL-6 upregulation of protein S in HepG-2 hepatoma cells was suppressed by the TNF agonist, anti-TNF RI

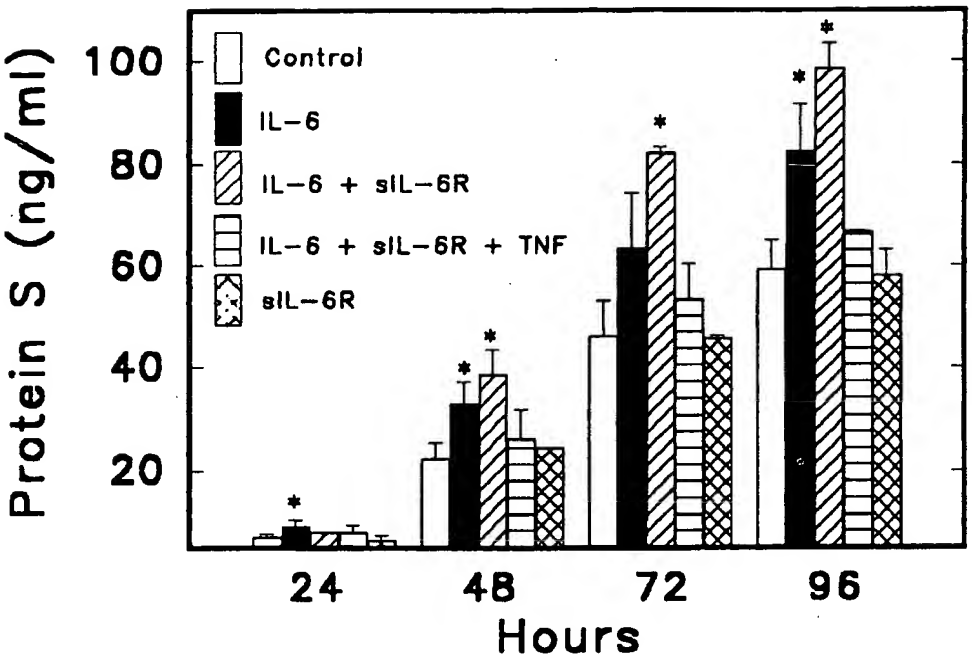


FIG. 3.

IL-6/sIL-6R upregulation of protein S in HepG-2 hepatoma cells was suppressed by TNF.

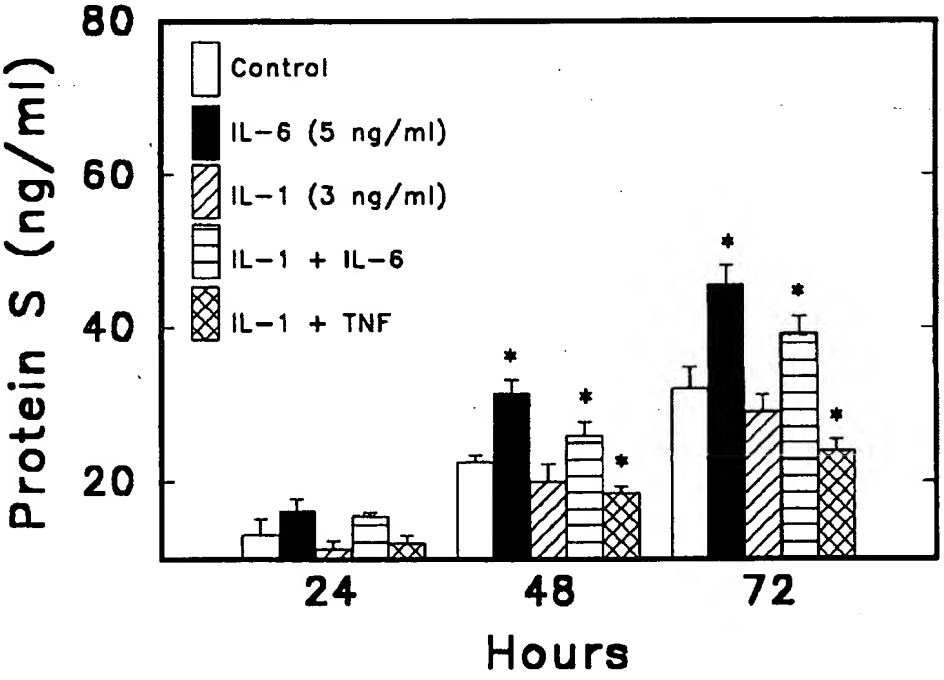


FIG. 4.

IL-1α partially blocked IL-6 upregulation of protein S and synergized with TNF-α to downregulate protein S in HepG-2 hepatoma cells.

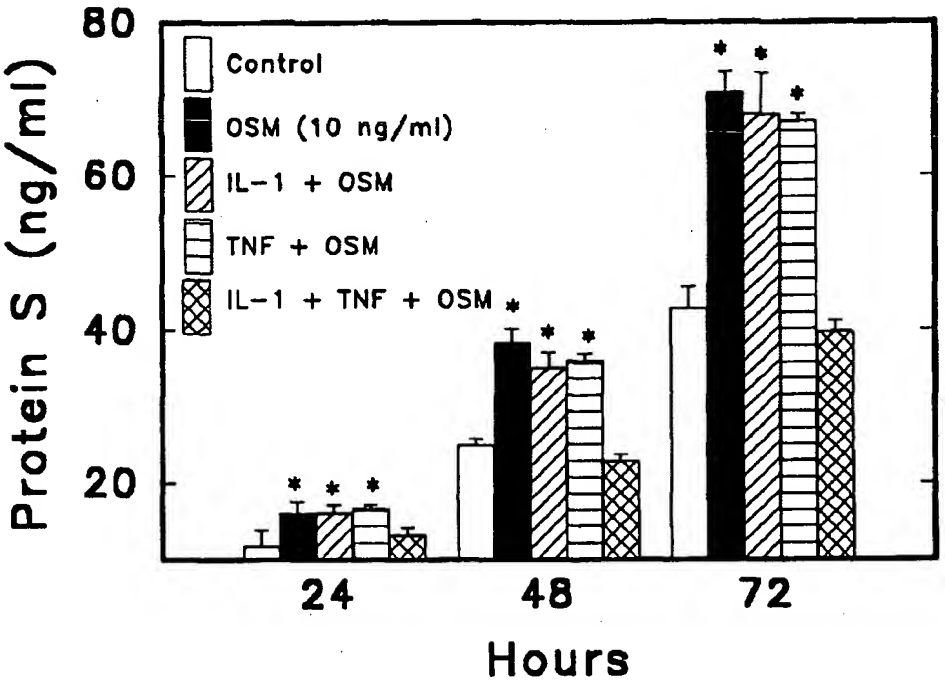


FIG. 5

OSM upregulation of protein S was blocked by the combination of IL-1α and TNF-α but not blocked by either when used separately with OSM.

Oncostatin M (OSM) upregulation of protein S was not effected by either IL-1α or TNF-α (Fig. 5). However, in combination, IL-1α and TNF-α synergized to suppress the OSM mediated upregulation of protein S to basal levels (Fig. 5). OSM in combination with IL-6 produced a modest but non-significant increase in protein S over that of OSM.

DISCUSSION

Activation of the coagulation cascade is associated with sepsis and disseminated intravascular coagulation (DIC), frequently occurs as a complication. Although the pathological mechanisms responsible for DIC are not clear, some data indirectly indicate that impairment of the protein C/protein S anticoagulant system may be a contributing factor (11-17,31,32). It has been previously shown that IL-6 upregulated protein S synthesis in HepG-2 cells (40), while TNF-α downregulated protein S production in endothelial cells (42). In the current study, we have investigated the combined effects of IL-1α, IL-6, TNF-α, and OSM on hepatic protein S production using the HepG-2 heptoma cells as a model. Our results show

that: 1) TNF-α significantly suppressed protein S upregulation by IL-6, but not by OSM; 2) the TNF-α agonist, anti-TNF RI monoclonal antibody, similarly suppressed IL-6 induced protein S upregulation; 3) the enhancement of the IL-6 effect by sIL-6R was suppressed by TNF-α; and 4) IL-1α was significantly less suppressive of protein S upregulation by IL-6 and OSM, and the combination of IL-1α and TNF-α synergized to totally suppress IL-6 mediated protein S upregulation.

Although IL-6 serum levels have been reported to be elevated in sepsis (43), its role is presently not clear. The acute-phase reaction, triggered by IL-6, is generally considered beneficial, having a protective role for the host (37-38). However, a recent report has indicated that IL-6 may play an indirect role in coagulation activation in sepsis (17). Our previous data, which reported IL-6 upregulation of both protein S and C4BP (40), did not address previously reported clinical observations of decreased free protein S levels that were concomitant with elevated C4BP levels. This apparent physiologic imbalance has been documented in some inflammatory conditions that had been associated with a predisposition to thrombotic episodes (5-7,15,44). The results presented here, together with our companion paper, has clarified this apparent paradox by demonstrating that TNF-α blocked the IL-6 upregulation of both protein S and C4BP. These findings were not surprising since it has been earlier shown that TNF-α inhibited the IL-6 upregulation of other hepatic proteins such as C-reactive protein and serum amyloid A in primary cultures of human hepatocytes (45) as well as fibrinogen and haptoglobin in HepG-2 cultures (46).

Oncostatin M has previously been reported to induce acute-phase proteins in the HepG-2 cells as well as in other hepatic derived cells (47). It has been suggested that OSM could regulate the in vivo acute-phase response in at least two ways, with neither mutually exclusive of the other; OSM could induce IL-6 production in endothelial cells, thereby increasing the levels of circulating IL-6 , or it could induce the acute-phase response by interacting directly with hepatocytes. The biological effects of OSM and IL-6 are very similar because they both share the gp130 component of the IL-6 receptor complex.

The observation that, in contrast to IL-6, TNF-α had no effect on oncostatin M mediated upregulation of protein S, though unexpected, could be due to several possibilities. For example, the negative effect of TNF-α may be mediated through ligand binding receptor pathways, such as the gp80 component of the IL-6 receptor. Alternatively, a post-receptor signalling pathway could be a target of TNF-α. OSM upregulation of protein S in the presence of TNF-α further illustrates the importance of functional redundancy in the cytokine network for maintaining homeostasis and host protection. However, TNF and IL-1 in combination could be an overriding event leading to a procoagulant condition. Taken together with our earlier data (40), these results indicate that perhaps the most important role of IL-6 during inflammation and sepsis is protective by eliciting the acute-phase response and maintaining a balance in the hepatic anti-coagulation proteins. Impairment of the protein C/protein S pathway may be a TNF-α and IL-1α mediated event that leads to a hemostatic imbalance favoring a procoagulant bias through downregulation of both endothelial cell protein S (42) and thrombomodulin (32). Since high levels of IL-6 in sepsis generally follows TNF-α, it can be argued that IL-6, in part, attempts to restore hemostatic balance.

[1]Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

REFERENCES

1. GRIFFIN, J.H., EVATT, B.L., ZIMMERMAN, T.S., KLEIS, A.J. and WIDEMAN, C. Deficiency of protein C in congenital thrombotic disease. J Clin Invest 68:1370-1373, 1981.
2. COMP, P.C., NIXON, R.R., COOPER, M.R. and ESMON, C.T. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest 74, 2082-2088, 1984.
3. SCHWARTZ, H.P., FISCHER, M, HOPMEIER, P., BATARD, M.A. and GRIFFIN, J.H., Plasma protein S deficiency in familial thrombotic disease. Blood 64, 1297-1300, 1984.
4. ENGESSER, L., BROEKMANS, A.W., BRIET, E., BROMMER, E.J. and BERTINA, R.M. Hereditary protein S deficiency: Clinical manifestations. Ann Int Med 106, 677-682, 1987.
5. VIGANO-D'ANGELO, S., D'ANGELO, A., KAUFMAN, C.E. Jr, SHOLER, C., ESMON, C.T. and COMP, P.C. Protein S deficiency occurs in the nephrotic syndrome. Ann Intern Med 107, 42-47, 1987.
6. D'ANGELO, A., VIGANO-D'ANGELO, S., ESMON, C.T. and COMP, P.C. Acquired deficiencies of protein S: Protein S activity during oral anticoagulation, in liver disease and in disseminated intravascular coagulation. J Clin Invest 81, 1445-1454, 1988.
7. MALM, J., LAURELL, M. and DAHLBÄCK, B. Changes in the plasma levels of vitamin K-dependent proteins C and S and of C4b-binding protein during pregnancy and oral contraception. Br J Haematol 68, 437-443, 1988.
8. RUIZ-ARGUELLES, G.J., RUIZ-ARGUELLES, A., DELEZE, M. and ALARCON-SEGOVIA, D. Acquired protein C deficiency in a patient with primary antiphospholipid syndrome. Relationship to reactivity of anticardiolipin antibody with thrombomodulin. J of Rheumatology 16, 381-383, 1989.
9. GEARSON, W.T., DICKERMAN, J.D., BOVILL, E.G. and GOLDEN, E. Severe acquired protein C deficiency in purpura fulminans associated with disseminated intravascular coagulation: treatment with protein C concentrate. Pediatrics 91, 418-1422, 1993.
10. STAHL, C.P., WIDEMAN, C.S., SPIRA, T.J., HAFF, E.C., HIXON, G.J. and EVATT, B.L. Protein S deficiency in men with long-term human immunodeficiency virus infection. Blood. 81, 1801-1807, 1993.
11. TAYLOR, F.B. Jr., CHANG, A., ESMON, C.T., D'ANGELO, A., VIGANO-D'ANGELO, S. and BLICK, C. E. Protein C prevents the coagulopathic and lethal effect of E. coli infusion in the baboon. J Clin Invest 79, 918-925, 1987.
12. HEEB, M.J., MOSHER, D. and GRIFFIN, J.H. Activation and complexation of protein C and cleavage and decrease of protein S in plasma of patients with intravascular coagulation. Blood 73, 455-461, 1989.
13. KOGAN, A.E. and STRUKOVA, S. M. Protein C decreases in experimental DIC in rats. Thromb Research 57, 825-826, 1990.

14. OKAJIMA, K., IMAMURA, H., KOGA, S., INOUE, M., TAKATSUKI, K. and AOKI, N. Treatment of patients with disseminated intravascular coagulation by protein C. Am J Hematol 33, 277-278, 1990.
15. TAYLOR, F., CHANG, A., FERRELL, G., MATHER, T., CATLETT, R., BLICK, K. and ESMON, C.T. C4b-binding protein exacerbates the host response to Escherichia coli. Blood 78, 357-363, 1991.
16. FOURRIER, F., CHOPIN, C., GOUDEMAND, J., HENDRYCX, S., CARON, C., RIME, A., MAREY, A. and LESTAVEL, P. Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. Chest. 101, 816-823, 1992.
17. van der POLL, T., LEVI, M., HACK, C.E., ten Cate, H., van DEVENTER, S.J.H., EERENBERG, A.J.M., de GROOT, E.R., JANSEN, J., GALLANTI, H., BÜLLER, H.R., ten Cate, J.W. and AARDEN, L.A. Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. J Exp Med 179, 1253-1259, 1994.
18. ESMON, C.T. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem 264, 4743-4746, 1989.
19. KISIEL, W., CANFIELD, W.M., ERICSSON, L.H. and DAVIE, E.W. Anticoagulant properties of bovine protein C following activation by thrombin. Biochemistry 16, 5824-5831, 1977.
20. WALKER, F.J., SEXTON, P.W. and ESMON, C.T. The inhibition of blood coagulation by activated protein C through selective inactivation of activated Factor V. Biochim Biophys Acta 571, 333-342, 1979.
21. WALKER, F.J. Regulation of activated protein C by protein S. J Biol Chem 256, 11128-11131, 1981.
22. ESMON, N.L., OWEN, W.G. and ESMON, C.T. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J Biol Chem 257, 859-864, 1982.
23. DAHLBÄCK, B. and STENFOLO, J. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci USA 78, 2512-2516, 1981.
24. DAHLBÄCK, B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. J Biol Chem 261, 12022-12027, 1986.
25. DAHLBÄCK, B., Protein S and C4b-binding protein: Components involved in the regulation of the protein C anticoagulant system. Thromb Haemost 66, 49-61, 1991.
26. BEVILACQUA, M.P., POBER, J.S., MAJEAU, G.R., FIERS, W., COTRAN, R.S. and GIMBRONE, M.A. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: Characterization and comparison with the actions of interleukin 1. Proc Nat Acad Sci USA 83, 4533-4537, 1986.
27. NAWROTH, P.P. and STERN, D.M. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 163, 740-745, 1986.
28. BEUTLER, B. and CERAMI, A. Cachectin. more than a tumor necrosis factor. N Engl J Med 316, 379-385, 1988.
29. WAAGE, A., BRANDTZAEG, P., HALSTENSEN, A., KIERULF, P. and ESPEVIK, T. The complex pattern of cytokines in serum from patients with

meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. J. Exp Med. 169, 333-338, 1989.

30. van DEVENTER, S.J.H., BULLER, H.R., ten CATE, J.W., AARDEN, L.A., HACK, C.E. and STURK, A. Experimental endotoxemia in humans:analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood 76, 2520-2526, 1990.
31. van der POLL, T., BÜLLER, H.A., ten CATE, H., WORTEL, C.H., BAUER, K.A., van DEVENTER, S.J.H., HACK, C.E., SAUERWEIN, H.P., ROSENBERG, R.D. and ten CATE, J.W. Activation of coagulation after administration of tumor necrosis factor to normal subjects. N Eng J Med 322, 1622-1627, 1990.
32. CONWAY, E.M. and ROSENBERG, R.D. Tumor necrosis factor suppresses transcription of the thrombomodulin gene in endothelial cells. Mol Cell Bio 8, 5588-5592, 1988.
33. SCARPATI, E.M. and SADLER, J.E. Regulation of endothelial cell coagulant properties: Modulation of tissue factor, plasminogen activator inhibitors, and thrombomodulin by phorbol 12-myristate 13-acetate and tumor necrosis factor. J Biol Chem 264, 20705-20713, 1989. Addendum 265, 14696, 1990.
34. TRACEY, K.J., BEUTLER, B., LOWRY, S.F., MERRYWEATHER, J., WOLPE, S., MILSARK, I.W., HARIRI, R.J., FAHEY III, T.J., ZENTELLA, A., ALBERT, J.D., SHIRER, G.T. and CERAMI, A. Shock and tissue injury induced by recombinant human cachectin. Science 234, 470-474, 1986.
35. SEHGAL, P.B., GRIENINGER, G. and TOSATO, G. Regulation of the Acute phase and Immune responses:Interleukin-6. Ann. New York Acad. Sci. 557-583, 1989.
36. HEINRICH, P.C., CASTELL, J.V. and ANDUS, R. Interleukin-6 and the acute phase response. Biochem J 265, 621-636, 1990.
37. GAULDIE, J, GEISTERFER, M., RICHARDS, C. and BAUMANN, H. IL-6 regulation of the hepatic acute phase response. In IL-6: Physiopathology and Clinical Potential. M. Revel, editor. Raven Press 88, 151-162, 1992.
38. KUSHNER, I. The role of IL-6 in regulation of the acute phase response. In IL-6: Physiopathology and Clinical Potential. M. Revel, ed. Raven Press 88,163-171, 1992.
39. TAYLOR, F.B., Jr., CHANG, A., RUF, W., MORRISSEY, J.H., HINSHAW, L., CATLETT, R., BLICK, K. and EDGINGTON, T.S. Lethal E. coli septic shock is prevented by blocking tissue factor with monoclonal antibody. Circ Shock 33, 127-134, 1991.
40. HOOPER, W.C., PHILLIPS, D.J., RIBEIRO, M., BENSON, J. and EVATT, B.L. IL-6 upregulates protein S expression in the HepG-2 hepatoma cell line. Thromb Haemost 73, 819-824, 1995.
41. PHILLIPS, D.J., EVATT, B.L. and HOOPER, W.C. Development of an ELISA for quantification of human protein S in cell culture fluids using commercial polyclonal antisera. J Immunoassay 15, 411-428, 1994.
42. HOOPER, W.C., PHILLIPS, D.J., RIBERIO, M.J.A., BENSON, J.M., GEORGE, V.G., ADES, E.W. and EVATT, B.L. Tumor necrosis factor-α downregulates protein S secretion in human microvascular and umbilical vein endothelial cells but not in the HepG-2 hepatoma cell line. Blood. 84, 483-489, 1994.

43. HACK, C.E., de GROOT, E.R., FELT-BERSMA, R.J.F., NUIJENS, J.H., STRACK van SCHIJNDEL, R.J.M., EERENBERG-BELMER, A.J.M., THIJS, L.G. and AARDEN, L.A. Increased plasma levels of interleukin-6 in sepsis. Blood 74, 1704-1710, 1989.
44. COMP, P.C., DORAY, D., PATTON, D. and ESMON, C.T. An abnormal plasma distribution of protein S occurs in functional protein S deficiency. Blood 67, 504-508, 1986.
45. YAP, S.H., MOSHAGE, H.J., HAZENBERG, B.P.C., ROELOFS, H.M.J., BIJZET, J., LIMBURG, P.C., AARDEM, L.A. and van RIJSWIJK, M.H. Tumor necrosis factor (TNF) inhibits interleukin (IL)-1 and/or IL-6 stimulated synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. Biochimica et Biophysica Acta 1091, 405-408, 1991.
46. MACKIEWICZ, A., SPEROFF, T., GANAPATHI, M.K. and KUSHNER, I. Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines. J Immunol 146, 3032-3027, 1991.
47. RICHARDS, C.D., BROWN, T.J., SHOGAB, M., BAUMANN, H. and GAULDIE, J. Recombinant oncostatin M stimulates the products of acute phase proteins in HepG-2 cells and rat primary hepatocytes in vitro. J Immunol 148, 1731-1736, 1992.

STIC-ILL

VAP W

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

363825

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

Thrombosis and Haemostasis – © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 69 (2) 164–172 (1993)

Inflammatory and Procoagulant Mediator Interactions in an Experimental Baboon Model of Venous Thrombosis

Thomas W. Wakefield[1], Lazar J. Greenfield[1], Mark W. Rolfe[2], Alphonse DeLucia III[1], Robert M. Strieter[2], Gerald D. Abrams[3], Steven L. Kunkel[3], Charles T. Esmon[4], Shirley K. Wrobleski[1], Amy M. Kadell[1], Marie D. Burdick[2], and Fletcher B. Taylor[4]

From the [1]Jobst Research Laboratories, Section of Vascular Surgery, Department of Surgery, [2]Department of Medicine, and [3]Department of Pathology, University of Michigan Medical Center, Ann Arbor, Michigan and the [4]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation and the Howard Hughes Medical Institute Research Laboratories, Oklahoma City, OK, USA

Summary

Theoretic and in vitro evidence suggests that thrombosis and inflammation are interrelated. The purpose of the present study was to define the relationship between inflammation and deep venous thrombosis (DVT) in an in vivo model. Initiation of DVT was accomplished by administration of antibody to protein C (HPC_4, 2 mg/kg) and tumor necrosis factor (TNF, 150 μg/kg); stasis; and subtle venous catheter injury. Thrombosis was assessed by thrombin-antithrombin assay (TAT), ^{125}I-fibrinogen scanning (scan) over both the proximal and distal iliac veins, and ascending venography. Cytokines TNF, interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and interleukin-8 (IL-8) were measured along with differential white blood cell counts, platelet counts, fibrinogen (FIB), and erythrocyte sedimentation rates (ESR). Baboon pairs were sacrificed on day 3 (T + 3d), T + 6d, and T + 9d and veins removed. All animals developed inferior vena cava and left iliofemoral DVT by venography; no right DVT was found. TAT was elevated by T + 1hr and peaked at T + 3hrs. Left iliofemoral DVT was found at T + 1hr by scan and reached a 20% uptake difference between the affected left and nonaffected right side at T + 3hrs. TNF peaked at T + 1hr; MCP-1 peaked at T + 6hrs; IL-8 and IL-6 peaked on T + 2d; all cytokines declined to baseline. TNF and TAT elevations were found to correlate with all cytokines; elevations in IL-8 were correlated with elevations in MCP-1 and IL-6 ($p < 0.05$). Correlation between cytokines and scan revealed a significant ($p < 0.05$) relationship only between elevations in IL-6 and distal iliac fibrin accumulation; no significant correlation was found between IL-8 and MCP-1 and scan. Increased mature polymorphonuclear leukocytes were found by T + 2d; immature forms were prominent at T + 3hrs, T + 6hrs, and T + 2d. Increased monocytes were noted by T + 4d; increased lymphocytes and platelets by T + 8d. ESR and FIB were elevated by T + 3d. Histopathologic study revealed venous inflammation at T + 3d, with beginning thrombus organization by T + 6d. MCP-1 localized to areas of thrombus and phlebitis. The development of DVT in this model involves inflammatory as well as coagulant activity. We conclude that this model allows studies on the role of inflammatory mediators in the development and natural history of DVT.

Introducti n

Venous thrombosis is treated primarily by anticoagulation when it occurs within the deep venous system. However, there is evidence to suggest that thrombosis and inflammation are closely interrelated. For example, it is known that tumor necrosis factor (TNF), a polypeptide inflammatory cytokine released in response to sepsis, downregulates thrombomodulin expression likely through endocytosis and degradation of this cell-surface receptor leading to a hypercoagulable state (1). Tumor necrosis factor also internalizes the surface cofactor protein S and decreases the amount of free protein S available to act as a cofactor for protein C. Additionally, TNF induces the expression of tissue factor on the surface of vascular endothelium and inhibits the fibrinolytic system by suppressing the release of tissue plasminogen activator and inducing the secretion of plasminogen activator inhibitor type 1 (2–7). Although in vivo TNF has been shown to increase circulating tissue plasminogen activator early after administration, there follows a rapid inhibition thereafter by an even greater rise in plasminogen activator inhibitor type 1 (8). Finally, TNF, by downregulating thrombomodulin, decreases the production of protein C which normally inhibits the plasminogen activator inhibitor, thus decreasing the fibrinolytic potential of the blood. We have previously reported a reproducible model of deep vein thrombosis in the baboon involving infusion of a combination of the inflammatory cytokine TNF, antibody to protein C (HPC_4), venous stasis and subtle venous injury (9–10). The purpose of the present investigation was to define the importance and interrelationship between inflammatory and thrombotic factors in this primate model of venous thrombosis which we believe closely simulates its clinical counterpart.

Materials and Methods

Six adolescent baboons, mean weight 4.9 kg, were studied in 3 pairs, each pair of which was sacrificed 2 days (T + 3d), 5 days (T + 6d), and 8 days (T + 9d) after reagent administration (Fig. 1). The animals were anesthetized with 16 mg/kg of ketamine hydrochloride IM (100 mg/ml, Parke-Davis, Morris Plains, NJ) and 0.5 to 1.0 ml of thiamylal-sodium IV (2% solution, Bio-tal, Boehringer Ingelheim Animal Health Inc., St. Joseph, MO) and then maintained on isoflurane (1.5%) and oxygen anesthesia. The animals were hydrated with lactated Ringers solution (10 ml kg^{-1} h^{-1}) and subjected to a cut-down in the left femoral region for placement of a polyethylene catheter (PE 90) into the left superficial femoral vein with venous ligation distally and a second catheter into the left superficial femoral artery with ligation distal to the deep femoral artery. These catheters were used for the measurement of hemodynamic parameters, blood withdrawal, and venographic evaluation. Continuous wave doppler evaluation revealed continued arterial flow distally via the deep femoral artery. The venous catheter was placed so that its tip was located just at the level of insertion for initial venography and then it was advanced to the level of the renal veins for the next 6 h.

After baseline stabilization, HPC_4 was administered intravenously (IV) over 5 min at a dose of 2 mg/kg and then 30 min later, TNF was given IV

Correspondence to: Dr. Thomas W. Wakefield, University of Michigan Medical Center, 2210 THCC, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0329, USA

at a dose of 150 μg/kg over 3 min. HPC_4 (Charles Esmon, Oklahoma Medical Research Foundation, Oklahoma City, OK) is a monoclonal antibody IgG1 that binds to an epitope that overlaps the activation cleavage site on protein C. The binding of this antibody is dependent on Ca^{2+} and this antibody does not bind either the activation peptide or activated protein C itself. HPC_4 is isolated from mouse ascites fluid by ammonium sulfate fractionation (50%), desalting, and affinity chromatography on a 12-residue peptide corresponding to the epitope linked to affisel 15 at a concentration of 1 mg/ml. The column is extensively washed with 0.4 M NaCl, 0.02 M Tris-HCl, and 2 mM Ca^{2+}, pH 7.5. The column is eluted with 2 M NaCl, 1.5 M guanidine HCl, 2 mM EDTA, pH 7.5, and is then immediately desalted on a G-75 column into the appropriate buffer. The HPC_4 corresponds to the last major protein peak to elute from the column. Antibody yields are approximately 1 g from the 100 ml column, and yields from the ascites are approximately 7 mg/ml. Recombinant TNF (Cetus, Emeryville, CA) has a bioactivity of 6.5×10^6 units/0.3 mg, is 95% pure by SDS/PAGE, with an endotoxin concentration <0.058 ng/0.3 mg.

The thrombotic state was assessed by thrombin-antithrombin (TAT) assay (Enzygnost TAT, Behring, Sommerville, NJ) measuring absorbance at 492 nm of a chromogen (o-phenylenediamine dihydrochloride) using peroxidase-conjugated antibodies to the antithrombin III portion of TAT by a sandwich ELISA. ^{125}I-fibrinogen scanning and ascending venography also were used to document location and extent of thrombosis. Prior to reagent administration, ascending venography was performed through the catheter placed into the left superficial femoral vein and from a right saphenous vein percutaneous puncture using 10 ml iothalamate meglumine (60% Conray, Malinckrodt, St. Louis, MO). Next, 5 μCi ^{125}I-fibrinogen (ICN Biomedical, Irving, CA) was administered IV and external scanning was performed over the IVC and at two locations over each of the iliac veins as assessed from venography (right and left proximal, right and left distal) with a low energy gamma scintillation probe (Bicron Corp, Newbury, OH) accumulating counts for 60 s with a digital scaler for each measurement. Urine was aspirated before these measurements were made in order to correct for overlying bladder activity. Scanning was quantitated by determining the ratio of left sided to right sided counts at baseline before thrombus induction and then for each experimental time point, the ratio was corrected for baseline activity. Since there was a baseline non-thrombosed sample time point to which experimental activity could be compared to, normalization over the heart was not performed. A level of 20% difference between one iliac vein and the contralateral iliac vein was considered diagnostic of deep venous thrombosis (DVT), while counts over the IVC were used to determine the decay and elimination of ^{125}I-fibrinogen over time.

For the next 6 h, ^{125}I-fibrinogen scanning was performed hourly and ascending venography was performed 5 h after reagent administration (T + 5hrs). Additionally, the animals were monitored for their physiologic response to the infused reagents and thigh/calf girths were measured hourly. Scanning and thigh girth measurements continued daily until sacrifice (Fig. 1). Hematologic measurements obtained at baseline, 3 h after reagent administration (T + 3hrs), 6 h after reagent administration (T + 6hrs), and then daily until sacrifice included differential white blood cell and platelet counts measured by hand hemocytometer methods, fibrinogen (American Dade, Aguada, Puerto Rico), erythrocyte sedimentation rates (Fisher Scientific), and qualitative fibrin split products (American Dade, Aguada, Puerto Rico). During the first experimental day, blood was withdrawn from the indwelling venous catheter. After the first day, blood was withdrawn through a butterfly catheter from the right common femoral vein. TAT assays and cytokine levels were obtained at T + 3hrs, T + 6hrs, and daily until sacrifice and also included a sample at T + 1hr for two pairs of baboons. Cytokines measured included TNF, interleukin-8 (IL-8), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1).

Tumor necrosis factor bioassay. TNF bioactivity was measured using a semi-automated WEHI 164 subclone 13 fibrosarcoma lytic assay (11). Briefly, WEHI cells were suspended at a concentration of 5×10^4 cells in 100 ml of RPMI-1640, 10% FCS, 1 mM glutamine, 0.5 mg/ml actinomycin D, and plated in 96-well culture plates. Samples were serially diluted and 100 μl added to each well. Plates were incubated for 20 h at 37° C in 95% air and 5% CO_2. This was followed by the addition of 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5 dephenyltetrazolium bromide (MTT, 5 mg/ml; Sigma Chemical, St. Louis, MO) for an additional 4 h. One hundred fifty μl of supernatant fluid was removed from each well and replaced with 100 μl of isopropanol acidified with 0.04 M HCl (3.4 μl/ml isopropanol) to dissolve the tetrazolium crystals. Culture plates were read in a micro ELISA reader at 540 nm. Units of TNF were defined using an internal standard of human recombinant TNF with a specific activity of 22 units/ng protein. Rabbit anti-human TNF-α neutralizing antibody was used to establish the specificity of TNF induced cell lysis. This bioassay consistently detected TNF concentrations above 10 pg/ml.

Interleukin-8 ELISA. Immunoreactive IL-8 was quantitated using a modification of a double ligand method as previously described (12). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with 50 μl/well of rabbit anti-IL-8 antibody (1 ng/μl in 0.6 M NaCl, 0.26 M H_3BO_4, and 0.08 N NaOH, pH 9.6) for 16 h at 4° C and then washed with phosphate buffered saline (PBS), pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37° C. Plates were rinsed four times with wash buffer and diluted (neat, 1:5, and 1:10) plasma (50 μl) in duplicate were added, followed by incubation for 1 h at 37° C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit anti-IL-8 antibody (3.5 ng/μl in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS), and plates incubated for 30 min at 37° C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 min at 37° C. Plates were again washed four times and chromogen substrate (Bio-Rad-Laboratories, Richmond, CA) added. The plates were then incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 M H_2SO_4 solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant 72 amino acid IL-8, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected IL-8 concentrations above 10 pg/ml. The antibodies of this ELISA detect both the 72 and 77 amino acid forms of IL-8 as determined by Western blot analysis.

Interleukin-6 bioassay. IL-6 levels were measured in a bioactivity assay using the IL-6 dependent murine hybridoma cell line B13.29, clone B9 (provided by J. Gauldie, McMaster University, Hamilton, Ont.) (13). Briefly, serial dilutions of test samples were incubated with 100 μl of IL-6 dependent plasmacytoma cells at a concentration of 5×10^4 cells/ml for 72 h in a humidified incubator at 37° C and 5% CO_2. Proliferation was measured in a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,3 diphenyltetrazolium bromide (MTT, Sigma Chemical Co, St. Louis, MO). During the final 6 h of the plasmacytoma assay, 20 μl of MTT (5 mg/ml in PBS) was added to each sample. The culture medium was then aspirated and 100 μl of isopropanol acidified with 0.04 M HCl (3.4 μl/ml isopropanol) to dissolve the tetrazolium crystals was added. Absorbance at 550 nM was then measured. Serial dilutions of human rIL-6 (R & D Systems, Inc., Minneapolis, MN) were used to generate a standard curve for each assay. IL-6 concentrations in the experimental samples were calculated using the developed standard curves.

Monocyte chemotactic protein-1 ELISA. Antigenic MCP-1 was quantitated using a modification of a double ligand method as previously described (14). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with 50 μl/well of rabbit anti-MCP-1 antibody (1 ng/μl in 0.6 M NaCl, 0.26 M H_3BO_4, and 0.08 N NaOH, pH 9.6) for 16 h at 4° C and then washed with PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37° C. Plates were rinsed four times with wash buffer and diluted (neat, 1:5, and 1:10) plasma (50 μl) in duplicate were added, followed by incubation for 1 h at 37° C. Plates were washed four times with 50 μl/well biotinylated rabbit anti-MCP-1 (3.5 ng/μl in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS) added, and plates incubated for 30 min at 37° C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates incubated for 30 min at 37° C. Plates were washed four times and chromogen substrate (Bio-Rad Laboratories, Richmond, CA) added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 M H_2SO_4 solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant MCP-1 from 1 pg/ml to 1,000 ng/ml. This ELISA method consistently detected MCP-1 concentrations in a linear fashion greater than 30 pg/ml.

Sacrifice and collection of tissues for histopathologic exam. Animals were given ketamine at the same dose on a daily basis until sacrifice for purposes of blood withdrawal, radioactive fibrinogen scanning, and periodic ascending venography. Animal pairs were sacrificed on T + 3d

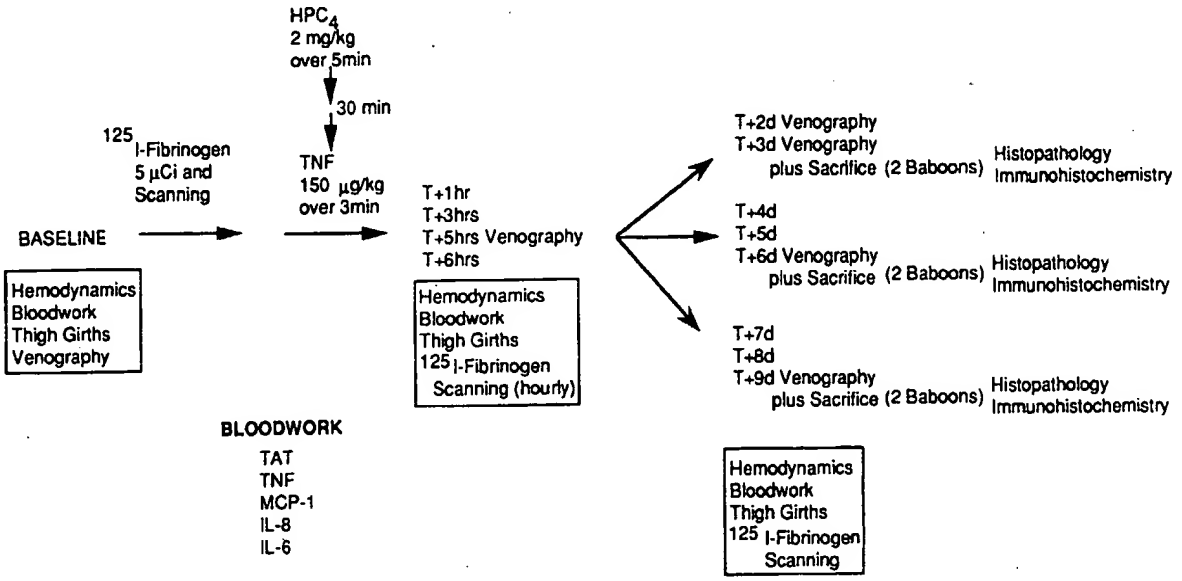


Fig. 1 Experimental protocol

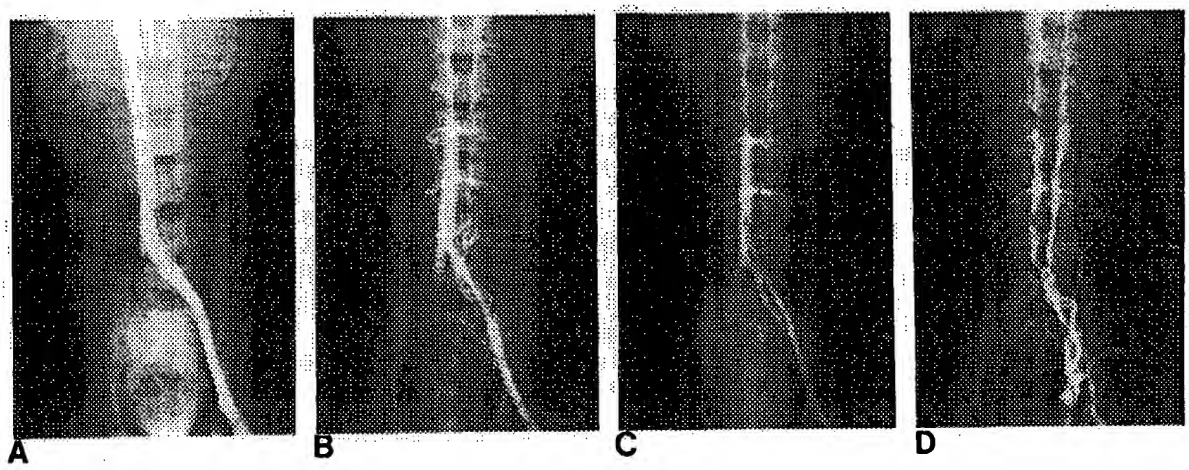


Fig. 2 Venography for baboon #3; (A) base, (B) T + 5hrs, (C) T + 2d, and (D) T + 9d

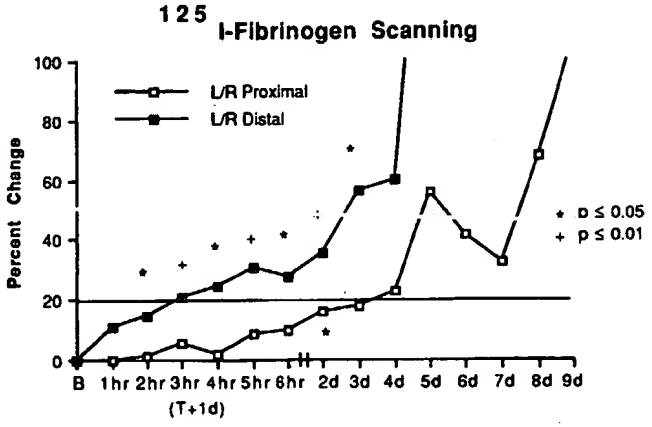


Fig. 3 ^{125}I-fibrinogen scanning; significant differences are from baseline

(48 h after reagent administration), T + 6d (5 days after reagent administration), and T + 9d (8 days after reagent administration). At sacrifice, the retroperitoneum was carefully dissected and the inferior vena cava and iliofemoral venous systems removed and placed in 10% neutral buffered formalin. The baboons received 1,000 IU heparin just prior to sacrifice in order to prevent the development of acute clot during the vein harvest. Serial segments of the vena cava and iliac veins were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic analysis. Portions of the IVC, right iliac vein, and left iliac vein were also evaluated with immunohistochemical staining for cell-associated MCP.

MCP-1 immunohistochemical staining. Paraffin embedded tissues were deparaffinized with xylene and rehydrated with serial dilutions of ethanol. Immunohistochemical staining was carried out using an improved biotin streptavidin amplified detection system with streptavidin-conjugated alkaline phosphatase (Biogenex Laboratories, San Ramon, CA). The tissue was blocked for non-specific binding using a 1:50 dilution of normal goat serum incubated for 1 h. Either pre-immune rabbit serum or immune polyclonal anti-human monocyte chemotactic peptide-1 serum at a

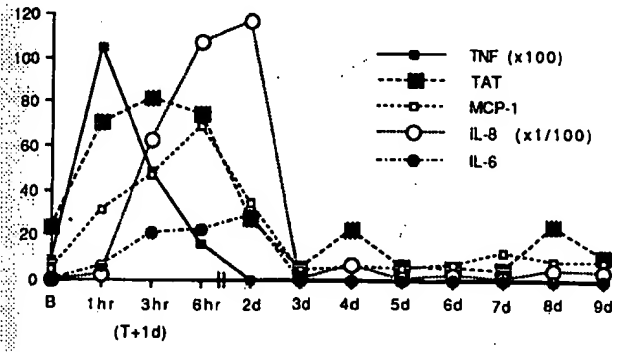


Fig. 4 Cytokine levels and TAT complexes

dilution of 1:500 were used to subsequently incubate the tissue for one hour. Slides were washed in 1 × PBS for 5 min and then a goat anti-rabbit IgG biotinylated antibody at a concentration of 1:35 was incubated with the tissue for 20 min. The tissue was washed for 10 min in 1 × PBS and then incubated with streptavidin-conjugated alkaline phosphatase at 1:35 concentration for 20 min. The tissue was washed again for 5 min in 1 × PBS and the substrate solution Fast Red applied. The reaction was extinguished after 20 min and the tissue counterstained for 4 min with 0.1% Mayer's hematoxylin. MCP-1 staining was qualitatively analyzed on a 0, 1+, 2+ and 3+ scale (0 = no staining, 3+ = maximal staining).

Statistical analysis included means ± SE mean. Correlations were performed using the non-parametric Kendall coefficient (tau) analysis. Animal care complied with the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publ No. 85-23, Revised 1985).

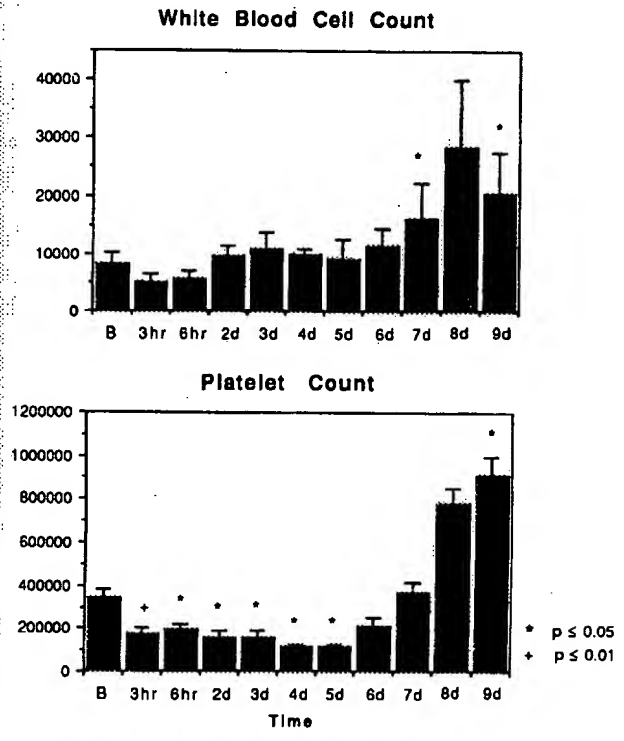


Fig. 5 White blood cell and platelet count analysis

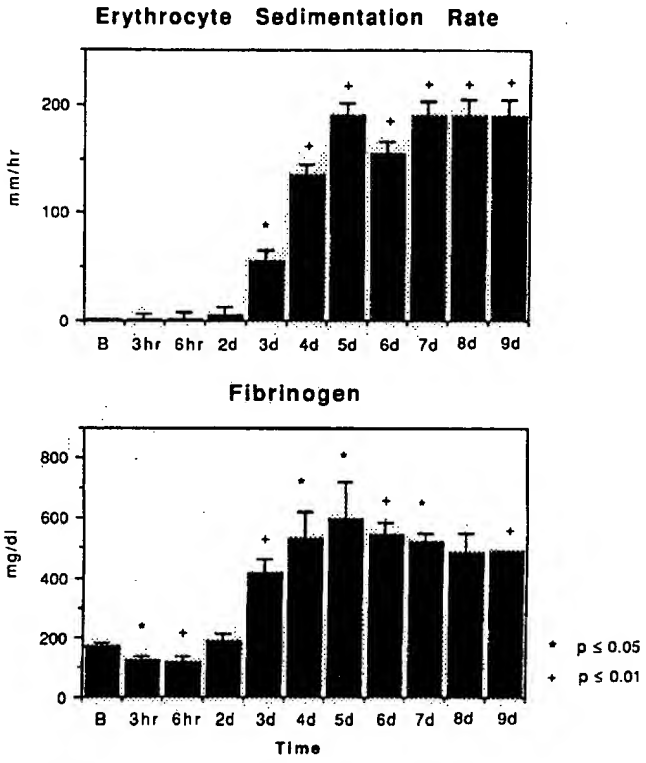
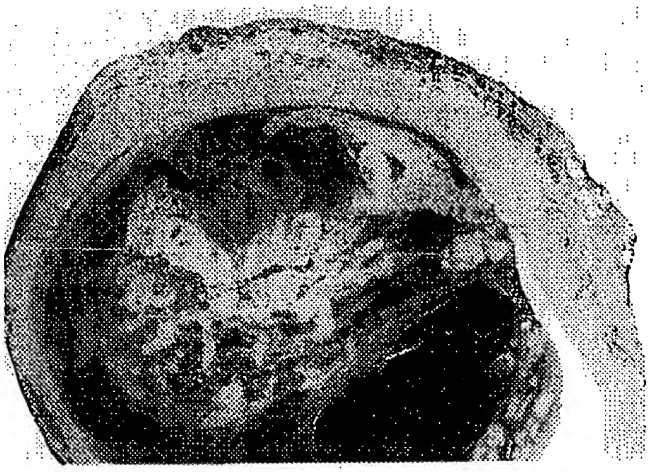


Fig. 6 Erythrocyte sedimentation rate and fibrinogen analysis

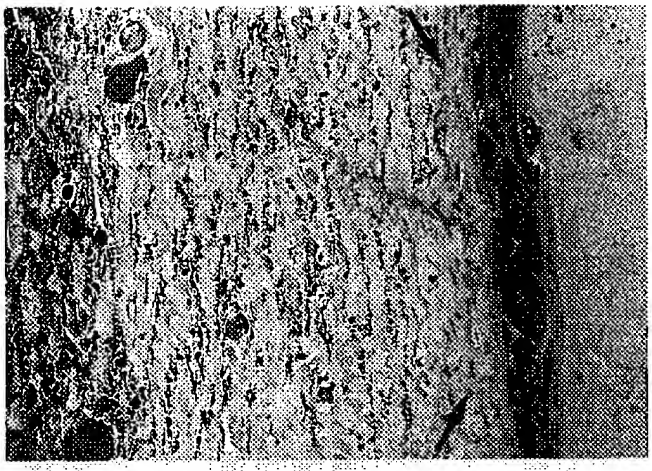
Table 1 Cytokine levels

	Baseline	T + 1hr	T + 3hrs	T + 6hrs	T + 2d	T + 3d
MCP-1 (ng/ml)	4.3 ± 2.8 (n = 6)	30.9 ± 11.5* (n = 4)	46.9 ± 14.6* (n = 6)	68.4 ± 15.8+ (n = 6)	34.2 ± 7.9+ (n = 6)	4.9 ± 2.1 (n = 6)
IL-8 (ng/ml)	0.07 ± 0.05 (n = 6)	0.02 ± 0.01 (n = 4)	0.62 ± 0.35 (n = 6)	1.07 ± 0.55 (n = 6)	1.16 ± 0.07 (n = 6)	0.01 ± 0.01 (n = 6)
IL-6 (ng/ml)	0.01 ± 0.01 (n = 6)	6.1 ± 3.7 (n = 4)	21.4 ± 5.3+ (n = 6)	22.2 ± 4.7+ (n = 6)	28.6 ± 20.6 (n = 6)	0.7 ± 0.5 (n = 6)
	T + 4d	T + 5d	T + 6d	T + 7d	T + 8d	T + 9d
MCP-1 (ng/ml)	6.5 ± 4.6 (n = 4)	5.5 ± 3.3 (n = 4)	6.7 ± 3.9 (n = 4)	12.8 ± 0.8 (n = 2)	8.3 ± 1.1 (n = 2)	8.3 ± 2.2 (n = 2)
IL-8 (ng/ml)	0.07 ± 0.07 (n = 4)	0.01 ± 0.00 (n = 4)	0.02 ± 0.02 (n = 4)	0.01 ± 0.01 (n = 2)	0.05 ± 0.05 (n = 2)	0.04 ± 0.04 (n = 2)
IL-6 (ng/ml)	0.4 ± 0.2 (n = 4)	0.1 ± 0.1 (n = 4)	0.00 ± 0.00 (n = 4)	0.00 ± 0.00 (n = 2)	0.00 ± 0.00 (n = 2)	0.00 ± 0.00 (n = 2)

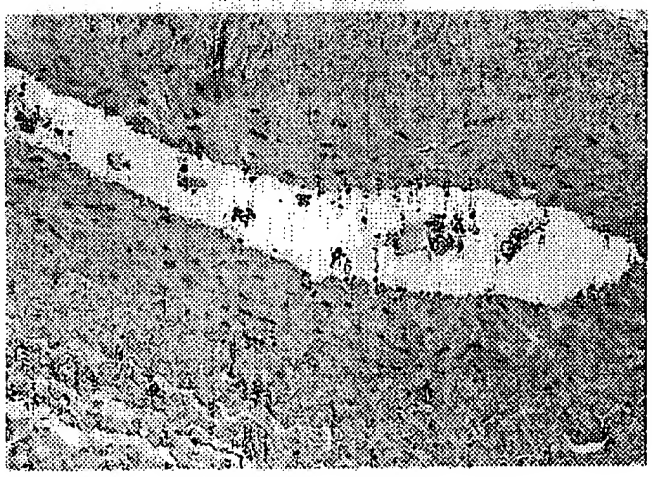
* $p \leq 0.05$. + $p \leq 0.01$.



A



B



C

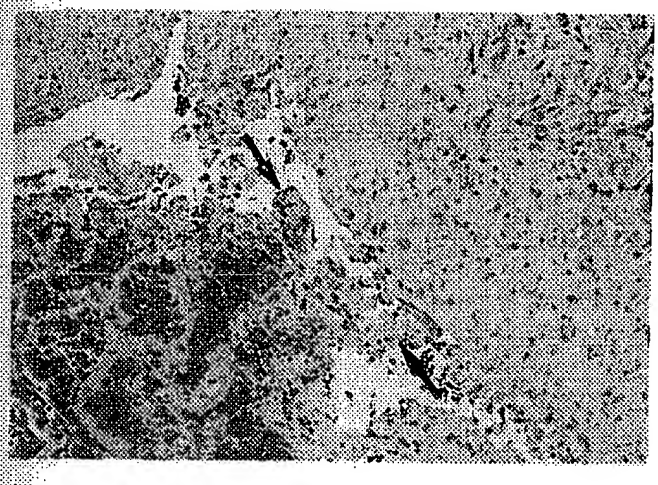
Fig. 7 (A) Vena cava, T + 3d. The lumen contains fresh thrombus adherent to the vessel. The space between the thrombus and the vessel wall on the right is shrinkage artifact (hematoxylin and eosin, 30×). (B) Vena cava, T + 3d. The dark nuclei within the wall are largely those of neutrophils, somewhat more concentrated in the zone adjacent to the lumen between the arrows. Thrombus is located to the right (hematoxylin and eosin, 235×). (C) Proximal right iliac vein, T + 3d. The lumen of the vessel is patent and the wall is devoid of inflammatory infiltrate. Compare with 7B (hematoxylin and eosin, 235×)

Results

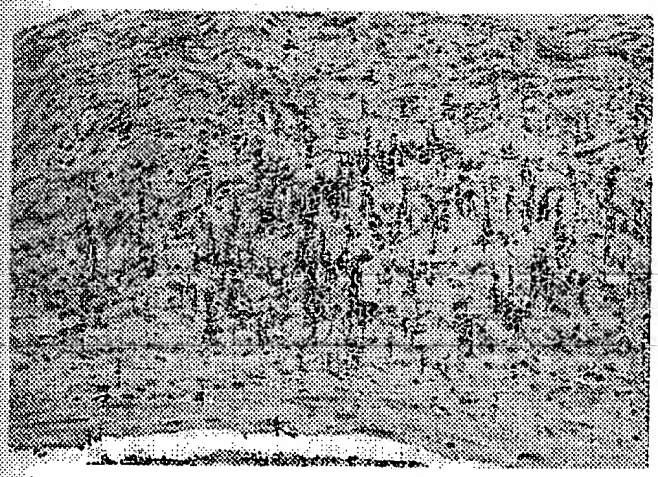
All animals developed evidence of inferior vena cava and left iliofemoral deep venous thrombosis (DVT) by venography, while no DVT was found on the right side (Fig. 2). Thrombus was indicated by filling defects and the presence of extensive collateral formation, noted radiographically by T + 5hrs. TAT complex elevation was noted as early as 1 h after reagent administration, peaked at T + 3hrs (base 23 ± 8 ng/ml; T + 1hr 70 ± 2 ng/ml, p <0.005; T + 3hrs 81 ± 11 ng/ml, p = 0.01), and decreased to baseline by T + 2d. ^{125}I-fibrinogen scanning revealed early thrombus formation. A difference between the affected left side and the nonaffected right side at the distal iliac vein location was found as early as T + 1hr (10.9%) and reached the 20% diagnostic level by T + 3hrs (20.9%, p <0.01). At the proximal iliac vein location, a difference became apparent by T + 3hrs (5.6%) and reached the diagnostic level by T + 4d (22.6%, Fig. 3). As verification of the scanning technique, internal counts with the probe directly placed over the proximal and distal iliac vein locations and the inferior vena cava were compared to external counts taken just prior to sacrifice in the last pair of baboons at the time of sacrifice. Comparison between internal and external counts revealed excellent correlation with an r = 0.88, p <0.05 for 12 observations. Animals displayed a mean maximal increase in thigh and calf girths of 1.4 ± 0.3 cm and 1.4 ± 0.3 cm on the affected left side versus 0.5 ± 0.2 cm and 0.7 ± 0.1 cm on the unaffected right side (p <0.05 for thigh measurements, left side compared to right side).

TNF levels peaked as early as T + 1hr and then rapidly declined (base 8.6 ± 8.3 ng/ml; T + 1hr 10.5 ± 3.3 × 10^3 ng/ml, p = 0.05, Fig. 4). These TNF levels are approximately 5-fold higher than would be expected based on the amount of TNF administered, suggesting TNF-induced TNF production. Levels of MCP-1 peaked at T + 6hrs; IL-8 and IL-6 peaked on T + 2d; all cytokines declined to basal levels by T + 3d (Fig. 4, Table 1). Cytokine levels were correlated to each other and to ^{125}I-fibrinogen scanning over the first 3 days of the study. Increased levels of TNF correlated significantly with elevated levels of IL-6 (tau 0.27, p <0.05) and MCP-1 (tau 0.23, p <0.05) while elevations in TNF correlated in a negative fashion to ^{125}I-fibrinogen scanning (proximal and distal iliac tau −0.24 and −0.28, p <0.05) suggesting that the elevations in TNF preceeded thrombosis. TAT complex elevations likewise correlated in a significant fashion to elevations in IL-8 (tau 0.27, p <0.05), IL-6 (tau 0.34, p <0.01) and MCP-1 (tau 0.42, p <0.01), although TAT levels were not found to correlate with fibrinogen scanning. Elevations in IL-8 correlated with increases in MCP-1 (tau 0.28 p <0.05) and IL-6 (tau 0.30, p <0.05). Finally and most importantly, only elevations in IL-6 were found to correlate in a positive fashion with ^{125}I-fibrinogen scanning (distal iliac, tau 0.24, p <0.05).

Differential white cell whole blood counts (WBC) revealed significant mature polymorphonuclear leukocyte (PMN) elevation by T + 2d; immature forms were prominent at T + 3hrs, T + 6hrs, and T + 2d (9%, 10%, and 10%, respectively; base 1%). Monocyte increases were noted by T + 4d with increased lymphocytes and platelets by T + 8d. In fact, significant total white blood cell increases were noted at T + 7d (base 8,248 ± 1,864 cells/mm^3; T + 7d 16,188 ± 6,088 cells/mm^3, p <0.05) and T + 9d (20,513 ± 6,858 cells/mm^3, p <0.05, Fig. 5). Increases in IL-8 correlated with elevations in immature PMNs (p <0.05) and monocytes (p <0.01) and declines in lymphocytes (p <0.05), while elevations in MCP-1 were correlated only with increasing monocytes (p <0.01). IL-6 elevations correlated with increases in monocytes (p <0.01) and declines in WBCs (p<0.01), PMNs (p <0.01), and lymphocytes (p <0.01) during the first 3 experi-

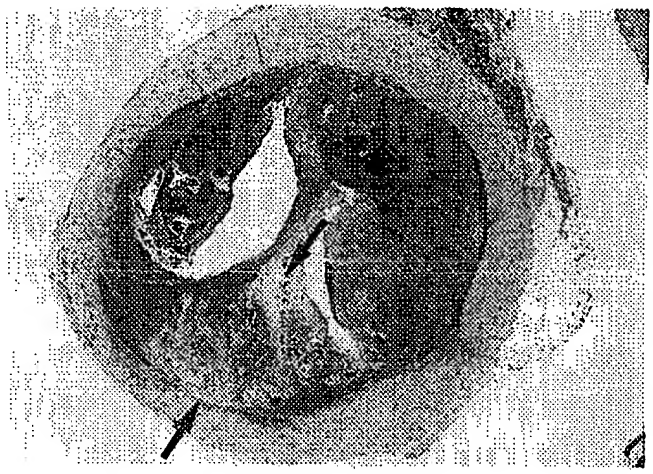


A



B

Fig. 8 (A) Left iliac vein, T + 6d. Within the lumen is the thrombus, with minimal evidence of organization at the right. Note beginning ingrowth of spindle-shaped cells between the arrows (hematoxylin and eosin, 235×). (B) Left iliac vein, T + 6d. A mixed inflammatory infiltrate is evident within the wall. Compare with 7B (hematoxylin and eosin, 235×)



A

B

Fig. 9 (A) Left iliac vein, T + 9d. Organization of the thrombus is advancing, as seen in the zone between the arrows (hematoxylin and eosin, 30×). (B) Left iliac vein, T + 9d. The light zone between the arrows represents organization of thrombus closest to the vein wall. Note the scattered inflammatory infiltrate within the wall itself (hematoxylin and eosin, 120×)

mental days. Platelet changes consisted of significant declines as early as T + 3hrs (base 339,833 ± 39,478/mm^3; T + 3hrs 169,667 ± 36,399/mm^3, $p < 0.01$) reaching a low of 113,000 ± 8,175/mm^3 at T + 4d ($p < 0.05$). A rebound elevation to 779,000 ± 73,000/mm^3 was noted by T + 8d (Fig. 5). There was an initial significant decrease in fibrinogen at T + 3hrs and T + 6hrs; erythrocyte sedimentation rates and fibrinogen levels both increased by T + 3d and remained elevated for the remainder of the experimental period, consistent with an acute phase response (Fig. 6; base fibrinogen 169 ± 15 mg/dl; peak 597 ± 121 mg/dl, $p < 0.05$). All animals displayed evidence of positive fibrin split products at dilutions of 1:20 to 1:160, usually beginning at T + 3hrs and extending throughout the study until T + 8d and T +9d.

In the animals sacrificed at T + 3d, fresh thrombus was found adherent to the walls of the inferior vena cava and left iliac vein (Fig. 7A). A neutrophilic infiltrate of somewhat irregular distribution was evident in the walls of these veins (Fig. 7B). The infiltrate was prominent in one animal and sparse in the second. At T + 6d, there was evidence of beginning organization of the thrombus in both animals (Fig. 8A). The inflammatory infiltrate at this stage, again of somewhat irregular distribution, included in addition to neutrophils mononuclear cells with oval vesicular nuclei, plump spindle cells, and occasional mitotic figures (Fig. 8B). At T + 9d, the appearance was similar but with more advanced organization of the thrombus (Figs. 9A, 9B). The right iliac veins, in contrast to the above, were generally unremarkable histologically at all intervals (Fig. 7C).

MCP-1 antigen immunolocalized to areas of thrombus and phlebitis histologically. All sections with overlying clot revealed either 2+/3+ or 3+/3+ MCP-1 staining while those sections with no clot revealed 0/3+ or 1+/3+ MCP-1 antigen (Fig. 10). Sections of left iliac vein examined in which clot was present on the section revealed either 2+/3+ or 3+/3+ MCP-1 staining while areas of IVC with clot all revealed 2+/3+ MCP-1 immunolocalization. Right iliac vein sections examined all showed either no MCP-1 (0/3+) or at most 1+/3+ MCP-1 staining (Table 2). However, one right iliac vein (T + 9d) with no evidence of overlying clot revealed 2+/3+ staining. This same vessel evaluated histologi-

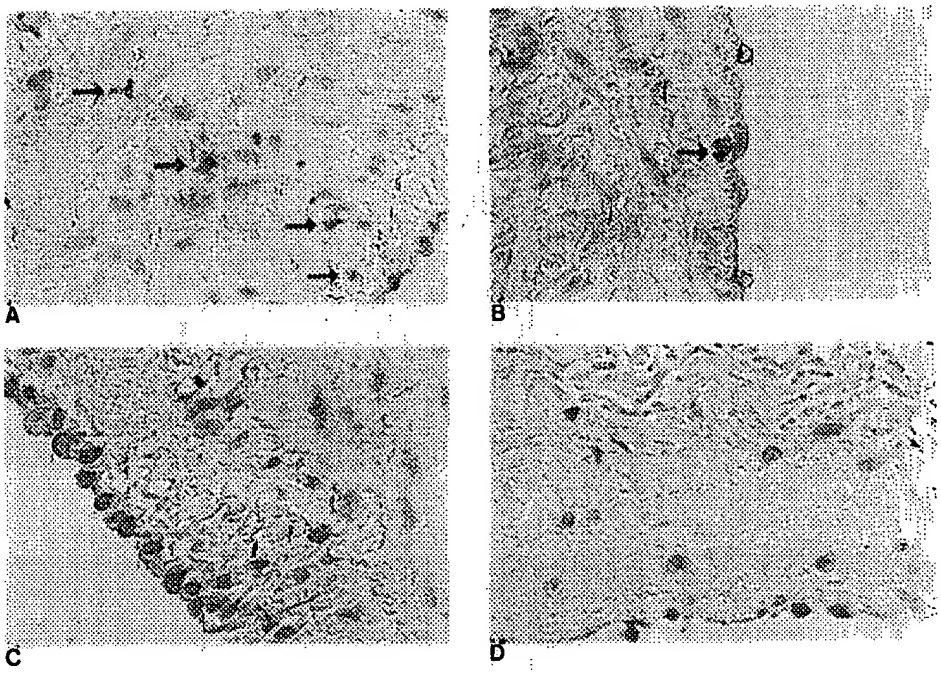


Fig. 10 Monocyte chemotactic protein-1 staining in (A) positive inferior vena cava at T + 3d, (B) positive left iliac vein at T + 3d, (C) unstained left iliac vein control at T + 3d, and (D) right iliac vein without thrombus at T + 3d, all sections under oil immersion. Note the granular appearance of the cell associated staining (arrows)

Table 2 Monocyte chemotactic protein-1 immunohistochemistry

Baboon	Right iliac		Left iliac		Distal IVC	
	CLOT	MCP	CLOT	MCP	CLOT	MCP
F91-1 (T + 6d)	–	+	+	++	+	++
F91-2 (T + 6d)	–	+	+	+++	+	++
F91-3 (T + 9d)	–	++	+	+++	+	++
F91-4 (T + 9d)	–	–	–	+	–	+
F91-5 (T + 3d)	–	+	+	+++	+	++
F91-6 (T + 3d)	–	–	+	+++	+	++

cally in a blinded fashion was the only right iliac vein to show evidence of inflammation.

Discussion

Evidence exists to suggest a link between the inflammatory and coagulant responses associated with thrombosis (15–24). This investigation was specifically designed to assess the role of inflammation in deep venous thrombosis in a well characterized primate model induced by the thrombogenic combination of reagents (TNF, HPC_4), stasis, and subtle venous injury.

The cytokines that were measured in this study are all mediators which have been found to be produced in endothelial cells in addition to inflammatory cells. IL-8 is produced from endothelial cells by both exogenous and endogenous stimuli while epithelial cells and fibroblasts can produce IL-8 only in response to host-derived cytokine stimuli (25). Thrombin generated on the surface of endothelial cells has also been found to be capable of activating neutrophil degranulation through IL-8 conversion (26), while TNF, IL-1 beta and LPS induce gene expression for IL-8 in human vascular smooth muscle cells in culture (27). The production of IL-8 may have a significant role in mediating neutrophil recruitment and activation in the procoagulant environment of endothelial cells exposed to thrombogenic stimuli. In a similar fashion, endothelial cells, vascular wall smooth muscle cells, epithelial cells and fibroblasts are all capable of making MCP-1 (27–29). In vitro, MCP-1 is expressed from endothelial cells in response to increasing doses of TNF, LPS and IL-1 beta. In fact, endothelial cells are unique with respect to expression of MCP-1 as these cells respond to exogenous LPS as well as endogenous TNF and IL-1 beta while epithelial cells and fibroblasts express MCP-1 only in response to host-derived cytokines (25). In the present study, both exogenous TNF and host-derived TNF (as indicated by the five-fold higher levels of measured than administered TNF) were presented to the venous endothelial cell surface. The production of MCP-1 may play a major role in the recruitment of monocytes and macrophages into the wall of the affected vein.

The production of IL-8 and MCP-1 from endothelial cells and vascular wall smooth muscle cells, both of which are exposed to the initial formation of thrombus, exposed to subsequent inflammatory stimuli, and active participants in all aspects of the coagulation response, suggests a strong association between thrombosis and inflammation. Neutrophils have been found to be the first cell line to adhere to the venous endothelium in a model of stasis-induced deep venous thrombosis in the cat and these neutrophils then stimulated thrombus formation (30). Leukocytes were found not only to adhere to the endothelium but to migrate under the endothelial cell layer, produce endothelial cell sloughing, and expose the basement membrane, producing a thrombogenic surface. Heparin was found to inhibit thrombus formation but not neutrophil adhesion in this model (31). Inflammatory cells in this model appear responsible not only for amplifying the thrombotic process but for initiating this response. Such a mechanism may play a similar role in our model system of venous thrombosis.

In vitro, IL-6 has likewise been produced from endothelial cells and vascular smooth muscle cells in response to a number of stimulating factors such as LPS, TNF, and IL-1 beta (32, 33). IL-6 stimulates the production of a full spectrum of acute phase proteins from hepatocytes such as C-reactive protein, serum amyloid, fibrinogen, alpha-1-antitrypsin, alpha-1-antichymotrypsin, and haptoglobin (34). We found that the elevation in IL-6, which was the last cytokine to rise, preceded the development of

the acute phase response as indicated by the elevation in erythrocyte sedimentation rate and fibrinogen. This elevation in IL-6 is clearly different than the release in IL-6 in patients given TNF, suggesting an effect of the thrombus itself and not solely the reagents (35). In addition, of all the cytokines measured, only IL-6 correlated with ^{125}I-fibrinogen scanning, a significant finding suggesting that IL-6 levels (as indicating a generalized inflammatory response) may be a good marker for the thrombotic process. ^{125}I-fibrinogen scanning revealed that thrombosis begins early after reagent administration (as early as 1 h) and reaches a diagnostic level by 3 h after reagent administration at the level of the distal iliac vein. As neutrophils have been shown to be activated as early as 30 min after TNF administration (35), the role of neutrophils in thrombus formation which can be detected as early as 1 h after reagent administration is suggested. Thrombin-antithrombin complex elevation alone does not necessarily signify venous thrombosis as TAT elevation is also noted in disseminated intravascular coagulation (DIC, 36). The initial decrease in fibrinogen and early appearance of positive fibrin split products suggest that in fact, a low-grade DIC is produced. Higher TAT levels have been noted clinically in patients with DIC (15.8 ng/ml) than in those with DVT (9.4 ng/ml). Peak levels in our study were much higher than these clinical values (peak 81.2 ng/ml) as were the base levels in the baboons (23.8 ng/ml). The reason for these high base TAT values is not known and may be species specific. However, relative to base values, peak TAT levels in our animals were approximately 3.9 × base levels while in patients with DVT, peak levels were approximately 4.5× base levels compared to 7.5 × base levels for DIC.

In this investigation, we have not addressed the possible role for even more proximal cytokine stimulation such as IL-1 beta or investigated the role of neutrophil adhesion molecules such as the LEC-CAM family or the integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18. IL-1 beta is expressed early during inflammation and has been found to be active on the cell surface of cytokine activated vascular smooth muscle cells in vitro (37), while the adhesion complex CD11/CD18 plays a major role in neutrophil adherence, neutrophil aggregation and eventual neutrophil-mediated injury (38). This activity may lead to autocrine and paracrine signaling in the vessel wall itself which potentiates the inflammatory response.

These observations suggest that inflammation and venous thrombosis can be linked. By this we mean that inflammation may influence whether a physiologic clot develops into a pathologic thrombus. Virchow's triad asserts that a change in the vessel wall coupled with changes in flow and changes in the coagulability of blood combine to produce thrombosis. In theory inflammation can mediate or extend two of the three limbs of this triad by promoting changes in the venous wall and the blood which favor growth of a physiologic clot into a pathologic thrombus. In constructing this baboon model we attempted to approximate those conditions by inducing an inflammatory response involving the vein wall with TNF, while increasing the coagulability of blood by inhibiting the protein C system with HPC_4. This, however, is insufficient without a change in flow and a nidus (subtle vein wall injury) for initial clot formation provided by the insertion of the catheter and ligature of the superficial femoral vein.

This paper is descriptive in that it presents a sequence of inflammatory and coagulant events associated with the development of the thrombus. The early rise of TNF at 60 min results from both the infusion and possibly the elaboration of endogenous TNF. This is followed closely by a rise in TAT which peaks at 180 min. Coincidental with this ^{125}I-fibrinogen scanning reveals deposition of fibrin in the distal vasculature at 60 min. It is possible that insertion of the catheter and infusion of the HPC_4 alone (without TNF) could induce the initial rise in TAT and early thrombosis. The fact, however, that other inflammatory mediators including MCP-1, IL-8 and IL-6 appear later and peak at approximately 360 min and 2 days, respectively, suggest a systemic inflammatory response that would not have come from insertion of the catheter and infusion of HPC_4 alone. Whether this inflammatory response contributes to the further development of the initial thrombus, though very likely, is not established by this association. The later appearance at 3 to 9 days of acute phase proteins, elevation in platelet counts, and increased sedimentation rate, suggests a role of IL-6 in the later stages of thrombus development. Further, the appearance of inflammatory leukocytes in the vein wall moving into the thrombus strongly suggests that over the 3 to 9-day period, the inflammatory and thrombotic processes along with the rise in acute phase proteins have become entertwined.

These results raise certain obvious questions, the first of which is at what point might the inflammatory component of this model play a role? Studies on the specific role of the thrombus as opposed to the administered TNF by infusing only HPC_4 or using a concomitant antibody to TNF would be helpful. Such studies would define the importance of the thrombus to the generation of both the local and systemic inflammatory response. In a similar fashion, such studies would clarify the role of TNF in not only initiating a portion of the inflammatory response but also in the generation of the thrombotic process as TNF in and of itself has been shown to release cytokines and promote thrombin formation (26–29, 39). Second, assuming it does play a role, at what point does the inflammatory process within the thrombus develop into either an uncontrolled response and phlegmasia or into a controlled resolution (would healing) recanalization response? Finally, can the inflammatory process be modified so as to limit both the thrombotic process and the destruction of the venous endothelial surface and the venous valves, a mechanism associated with the development of the chronic venous insufficiency syndrome. These questions require that further studies be performed in this model. Although this model requires the downregulation of natural anticoagulant mechanisms, it may be very similar to thrombosis in patients with antithrombin III deficiency or defective fibrinolysis or patients with normal anticoagulant mechanisms who sustain a strong ongoing inflammatory stimulus. The results of such analyses may have far-reaching implications for the manner in which deep venous thrombosis and/or prophylaxis is undertaken.

Acknowledgements

The authors wish to acknowledge the secretarial assistance of Cathy Blankenburg and the technical assistance of Joyce Klevering, Diane Smith, Linda Looby, Cami Zatkin, Melanie Payne and Pamela Lincoln.

REFERENCES

1. Esmon NL, Esmon CT. Protein C and the endothelium. Semin Thromb Hemostas 1988; 14: 210–5.
2. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 1986; 163: 740–5.
3. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci USA 1986; 83: 4533–7.
4. Conway EM, Bach R, Rosenberg RD, Konigsberg WH. Tumor necrosis factor enhances expression of tissue factor mRNA in endothelial cells. Thromb Res 1989; 53: 231–41.

5. Schleef RR, Bevilacqua MP, Sawdey M, Gimbrone MA Jr, Loskutoff DJ. Cytokine activation of vascular endothelium. Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor. J Biol Chem 1988; 263: 5797–803.
6. van Hinsbergh VW, Kooistra T, van den Berg EA, Princen HM, Fiers W, Emeis JJ. Tumor necrosis factor increases production of plasminogen activator inhibitor in human endothelial cells in vitro and in rats in vivo. Blood 1988; 72: 1467–73.
7. Medina R, Socher SH, Han JH, Friedman PA. Interleukin-1, endotoxin, or tumor necrosis factor/cachectin enhance the level of plasminogen activator inhibitor messenger RNA in bovine aortic endothelial cells. Thromb Res 1989; 54: 41–52.
8. Van der Poll T, Levi M, Büller HR, van Deventer SJH, de Boer JP, Hack CE, ten Cate JW. Fibrinolytic response to tumor necrosis factor in healthy subjects. J Exp Med 1991; 174: 729–32.
9. Wakefield TW, Wrobleski SK, Sarpa MS, Taylor FB Jr, Esmon CT, Chang A, Greenfield LJ. Deep venous thrombosis in the baboon: an experimental model. J Vasc Surg 1991; 14: 588–98.
10. Taylor FB, Esmon CT, Chang A. Primate model of deep vein thrombosis induced by inflammatory mediators [Abstract]. Circulation 1990; 82 (III): 770.
11. Strieter RM, Remick DG, Ham JM, Colletti LM, Lynch JP III, Kunkel SL. Tumor necrosis factor-alpha gene expression in human whole blood. J Leukocyte Biol 1990; 47: 366–70.
12. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JP III, Toews GB, Westwick J, Strieter RM. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. J Clin Invest 1990; 86: 1945–53.
13. Van Oers MH, Van der Heyden AA, Aarden LA. Interleukin 6 (IL-6) in serum and urine of renal transplant recipients. Clin Exp Immunol 1988; 71: 314–9.
14. Evanoff HL, Burdick MD, Moore SA, Kunkel SL, Strieter RM. A sensitive ELISA for the detection of human monocyte chemoattractant protein-1 (MCP-1). Immunol Invest 1992; 21: 39–45.
15. Taylor FB Jr, Chang AC, Esmon CT, Hinshaw LB. Baboon model of *Escherichia coli* sepsis: description of its four stages and the role of tumor necrosis factor, tissue factors, and the protein C system in septic shock. Curr Stud Hematol Blood Transfus 1991; 58: 8–14.
16. Voss BL, DeBault LE, Blick KE, Chang AC, Stiers DL, Hinshaw LB, Taylor FB Jr. Sequential renal alterations in septic shock in the primate. Circ Shock 1991; 33: 142–55.
17. Hinshaw LB, Archer LT, Beller-Todd BK, Coalson JJ, Flournoy DJ, Passey R, Benjamin B, White GL. Survival of primates in LD100 septic shock following steroid/antibiotic therapy. J Surg Res 1980; 28: 151–70.
18. Creasey AA, Stevens P, Kenney J, Allison AC, Warren K, Catlett R, Hinshaw LB, Taylor FB Jr. Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal *Escherichia coli*. Circ Shock 1991; 33: 84–91.
19. Taylor FB Jr, Chang A, Ruf W, Morrissey JH, Hinshaw L, Catlett R, Blick K, Edginton TS. Lethal *E. coli* septic shock is prevented by blocking tissue factor with monoclonal antibody. Circ Shock 1991; 33: 127–34.
20. Day KC, Hoffman LC, Palmier MO, Kretzmer KK, Huang MD, Pyla EY, Spokas E, Broze GJ Jr, Warren TG, Wun TC. Recombinant lipoprotein-associated coagulation inhibitor inhibits tissue thromboplastin-induced intravascular coagulation in the rabbit. Blood 1990; 76: 1538–45.
21. Taylor FB Jr, Chang AC, Peer GT, Mather T, Blick K, Catlett R, Lockhart MS, Esmon CT. DEGR-factor Xa blocks disseminated intravascular coagulation initiated by *Escherichia coli* without preventing shock or organ damage. Blood 1991; 78: 364–8.
22. Taylor F, Chang A, Ferrell G, Mather T, Catlett R, Blick K, Esmon CT. C4b-binding protein exacerbates the host response to *Escherichia coli*. Blood 1991; 78: 357–63.
23. Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. J Clin Invest 1987; 79: 918–25.
24. Taylor FB Jr, Emerson TE Jr, Jordan R, Chang AC, Blick KE. Antithrombin III prevents the lethal effects of *E. coli* infusion in baboons. Circ Shock 1988; 26: 227–35.
25. Kunkel SL, Standiford T, Metinko AP, Strieter RM. Endothelial cell-derived novel chemotactic cytokines. In: Lung Vascular Injury: Molecular and Cellular Response. Lefant C (ed). Marcel Dekker, Inc., New York 1991.
26. Hebert CA, Luscinskas FW, Kiely JM, Luis EA, Darbonne WC, Bennett GL, Liu CC, Obin MS, Gibrone MA Jr, Baker JB. Endothelial and leukocyte forms of IL-8. Conversion by thrombin and interactions with neutrophils. J Immunol 1990; 145: 3033–40.
27. Wang JM, Sica A, Peri G, Walter S, Padura IM, Libby P, Ceska M, Lindley I, Colotta F, Mantovani A. Expression of monocyte chemotactic protein and interleukin-8 by cytokine-activated human vascular smooth muscle cells. Arterioscler Thromb 1991; 11: 1166–74.
28. Strieter RM, Wiggins R, Phan SH, Wharram BL, Showell HJ, Remick DG, Chensue SW, Kunkel SL. Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. Biochem Biophys Res Commun 1989; 162: 694–700.
29. Sica A, Wang JM, Colotta F, Dejana E, Mantovani A, Oppenheim JJ, Larsen CG, Zachariae CO, Matsushima K. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. J Immunol 1990; 144: 3034–8.
30. Schaub RG, Simmons CA, Koets MH, Romano PJ, Stewart GJ. Early events in the formation of a venous thrombus following local trauma and stasis. Lab Invest 1984; 51: 218–24.
31. Simmons CA, Burdick MD, Schaub RG. Heparin inhibits fibrin, but not leukocytes, in a model of deep-vein thrombosis. J Surg Res 1987; 43: 468–75.
32. Loppnow H, Libby P. Adult human vascular endothelial cells express the IL-6 gene differentially in response to LPS or IL-1. Cell Immunol 1989; 122: 493–503.
33. Loppnow H, Libby P. Proliferating or interleukin 1-activated human vascular smooth muscle cells secrete copious interleukin 6. J Clin Invest 1990; 85: 731–8.
34. Hirano T, Akira S, Taga T, Kishimoto T. Biological and clinical aspects of interleukin 6. Immunol Today 1990; 11: 443–9.
35. Van der Poll T, van Deventer SJH, Hack CE, Wolbink GJ, Aarden LA, Buller HR, ten Cate JW. Effects on leukocytes after injection of tumor necrosis factor into healthy humans. Blood 1992; 79: 693–8.
36. Hoek JA, Sturk A, ten Cate JW, Lamping RJ, Berenda F, Borm JJ. Laboratory and clinical evaluation of an assay of thrombin-antithrombin III complexes in plasma. Clin Chem 1988; 34: 2058–62.
37. Loppnow H, Libby P. Functional significance of human vascular smooth muscle cell-derived interleukin 1 in paracrine and autocrine regulation pathways. Exp Cell Res 1992; 198: 283–90.
38. Mileski W, Borgstrom D, Lightfoot E, Rothlein R, Faanes R, Lipsky P, Baxter C. Inhibition of leukocyte-endothelial adherence following thermal injury. J Surg Res 1992; 52: 334–9.
39. Van der Poll T, Büller HR, ten Cate H, Wortel CH, Bauer KA, van Deventer SJ, Hack CE, Sauerwein HP, Rosenberg RD, ten Cate JW. Activation of coagulation after administration of tumor necrosis factor to normal subjects. N Engl J Med 1990; 322: 1622–7.

Received June 22, 1992 Accepted after revision October 7, 1992

STIC-ILL

M

363828

Fr m: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
T : STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

PMID
8623512

Tumori, 82: 78-80, 1996

CHARACTERIZATION OF CANCER-RELATED DISSEMINATED INTRAVASCULAR COAGULATION IN RELATION TO TUMOR NECROSIS FACTOR-ALPHA BLOOD CONCENTRATIONS: POSSIBLE THERAPEUTIC ROLE OF PENTOXIFYLLINE

Paolo Lissoni [1], Antonio Ardizzoia [1], Sandro Barni [1], Salvatore Pittalis [2], Fausto Rossini [3], Alvaro Porta [4], Gabriele Tancini [1]

[1] Division of Radiation Oncology, [2] Laboratory of Analyses, [3] Division of Hematology, and [4] Second Division of Internal Medicine, San Gerardo Hospital, Monza (Mi), Italy

Aims and Background: **Preliminary experimental data suggest the involvement of tumor necrosis factor (TNF) in determining endothelial damage related to disseminated intravascular coagulation (DIC). The present study was performed to investigate TNF secretion in DIC occurring in metastatic solid tumor patients and to evaluate the possible therapeutic role of pentoxifylline, which has been proven to have a TNF-lowering activity.** *Methods:* **The study included 20 metastatic solid tumor patients who showed clinical and laboratory signs of DIC. Pentoxifylline was given orally at a dose of 1200 mg/day for 28 days.** *Results:* **Abnormally high levels of TNF were found in 13/20 patients, and mean TNF serum levels observed in patients were significantly higher than those seen in a control group of 50 healthy subjects. Fibrinogen plasma concentrations were low in 11 cases. Patients with low fibrinogen values showed significantly higher mean TNF levels than those with normal or elevated concentrations. Pentoxifylline therapy induced a significant decrease in mean TNF concentrations and a significant increase in mean platelet number, which returned to within the normal range in 11/20 patients. An increase in platelets in response to pentoxifylline was more evident in patients with elevated pretreatment TNF values.** *Conclusions:* **Our results suggest the existence of abnormally high blood levels of TNF in cancer-related DIC, mainly in the presence of low fibrinogen values. Moreover, they indicate that pentoxifylline therapy may determine a decrease in TNF levels in DIC patients, an event associated with an increase in platelet number.**

Key words: disseminated intravascular coagulation, pentoxifylline, tumor necrosis factor-alpha.

One of the most severe complications of advanced tumors is disseminated intravascular coagulation (DIC) [3,5]. The mechanisms responsible for DIC in advanced solid tumor patients include two major events, consisting of endothelial cell damage and tissue injury [3,5]. Tissue injury occurs in solid tumors, whose cells produce procoagulant substances, generally tissue factors. In contrast, the causes responsible for endothelial damage with a subsequent increased endothelial procoagulant activity need to be better defined. Recent studies have shown that some cytokines, mainly tumor necrosis factor-alpha (TNF) [2,4,6], may enhance the procoagulant activity of endothelial cells, thereby suggesting an involvement of TNF in the pathogenesis of DIC-related endothelial damage in cancer. Moreover, TNF levels have appeared to be often elevated in advanced solid tumors [1], particularly in patients with cancer-related DIC [8]. At present, the only drug capable of inhibiting TNF secretion is pentoxifylline (PTX) [7], since corticosteroids have no effect [4]. Such a finding suggests a possible use of PTX to modulate TNF secretion in cancer patients.

The present study was performed to investigate TNF production in solid tumor-related DIC and the therapeutic effect of PTX.

Material and Methods

The study included 20 consecutive metastatic solid tumor patients (M:F, 7:13; median age, 53 years; range, 34-81) with clinical and laboratory signs of DIC. All patients had been previously treated with chemotherapy. The diagnosis of DIC was made according to the evidence of a rapid and ingravescent fall in platelet count associated with high serum levels of fibrinogen degradation products (FDP) and high plasma levels of X-degradation products (XDP). All patients showed a platelet count below 100,000/mm^3 before the onset of therapy. Routine laboratory tests were done at days 10, 7 and 3 prior to therapy, and at days 1, 3, 7, 14, 21 and 28 of PTX therapy. PTX was given orally at 400 mg thrice a day for at least 1 month. Eleven patients were treated concomitantly by chemotherapy. Moreover, all patients received supportive care consisting of fresh frozen plasma (3 units/week).

FDP and XDP were measured by the matex-particle agglutination assay. TNF serum levels were measured at days 1, 7 and 29 of PTX therapy by using an immunoradiometric assay and commercial kits (Medgenix Diagnostics, Brussels, Belgium). Intraassay and interassay coefficients of variation were below 4% and 5%, re-

To whom correspondence should be addressed: Paolo Lissoni, Divisione di Radioterapia, Ospedale San Gerardo, 20052 Monza (Mi), Italy. Tel. +39-39-2333681; fax +39-39-2333414.

Received July 27, 1994; accepted September 25, 1995.

spectively. Results were reported as mean ± SE. The control group consisted of 50 age-matched healthy subjects. Data were statistically analyzed by Student's *t* test, analysis of variance, coefficient of correlation, and the chi-squared test, as appropriate.

Results

Abnormally high pretreatment TNF levels were seen in 13 of 20 (65%) patients. Plasma fibrinogen values were low in 11, normal in 7, and paradoxically high in 2 patients. A negative correlation was seen between TNF and fibrinogen values (r = -0.7). As shown in Table 1, mean TNF concentrations were significantly higher in patients than in controls. Moreover, patients with an unknown primary tumor had significantly lower mean TNF levels than those affected by other histotypes. Finally, patients with low fibrinogen values had significantly higher mean TNF levels than those with normal or elevated fibrinogen concentrations. In contrast, no difference in mean liver enzyme serum levels was observed between patients with low or normal-high fibrinogen values (GOT, 58±7 vs 63±9 U/ml; GPT, 74±8 vs 69±11 U/ml; GGT, 267±38 vs 233±45 U/ml).

PTX induced a normalization of platelet count, with values above 100,000/mm^3, in 11 of 20 (55%) patients, and mean platelet number (n × 10^3/mm^3, mean ± SE) observed on PTX therapy was significantly higher than pretreatment values (before, 41±8; day 14, 84±12, P<0.05; day 21, 89±11, P<0.05; day 28, 109±13, P<0.01). Moreover, platelet normalization occurred significantly more often in patients with high pretreatment TNF levels than in those with normal TNF values (10/13 vs 1/7, P<0.01), whereas there was no difference between patients given or not given a concomitant chemotherapy (7/11 vs 4/9).

Table 1 - Clinical characteristics of 20 metastatic solid tumor patients with DIC and their individual TNF values in relation to fibrinogen levels and platelet number

Pat. no.	Sex	Age (yr)	Tumor histotype	TNF pg/ml	Fibrinogen mg/100 ml	Platelets n × 10^3/mm^3
1	M	52	Unknown primary	5	586	59
2	F	48	Unknown primary	10	280	46
3	F	39	Breast cancer	162	91	40
4	M	64	Colon cancer	64	112	67
5	F	71	Breast cancer	232	78	41
6	M	51	Unknown primary	7	315	28
7	F	48	Breast cancer	38	210	16
8	F	46	Unknown primary	10	97	5
9	F	67	Breast cancer	49	178	31
10	F	64	Breast cancer	187	89	39
11	F	59	Breast cancer	92	94	64
12	F	81	Soft tissue sarcoma	69	131	16
13	M	53	Unknown primary	4	620	26
14	M	56	Gastric cancer	35	128	66
15	F	49	Breast cancer	9	215	42
16	M	58	Unknown primary	31	236	58
17	M	61	Gastric cancer	41	194	36
18	F	72	Unknown primary	5	279	24
19	F	34	Soft tissue sarcoma	24	207	48
20	F	51	Breast cancer	36	175	9

Table 2 - Serum levels of TNF (mean ± SE) in DIC-related cancer in relation to the characteristics of the patients

Characteristic	No.	TNF (pg/ml)
Healthy controls	50	5 ± 2
Patients (before PTX therapy)	20	56 ± 14[1]
Patients with normal TNF	7	7 ± 1
Patients with high TNF	13	82 ± 19[2]
Patients with low fibrinogen	11	89 ± 22[3]
Patients with normal-high fibrinogen	9	15 ± 4
Unknown primary tumor	7	10 ± 4
Other tumor histotypes	13	79 ± 19[4]
After PTX therapy	20	18 ± 4[5]

PTX, pentoxifylline.
[1] P < 0.005 vs controls.
[2] P < 0.001 vs controls.
[3] P < 0.025 vs patients with normal-high fibrinogen levels.
[4] P < 0.01 vs patients with an unKnown primary tumor.
[5] P < 0.025 vs before PTX therapy.

Mean FDP and XDP values also significantly decreased on PTX therapy (FDP, 91±16 vs 178±22 μg/ml; XDP, 413±79 vs 1135±128 ng/ml, P<0.01). Finally, as shown in Table 2, mean TNF concentrations significantly decreased on PTX therapy, and the decline in TNF was negatively correlated with the increase in platelet number (r = -0.7).

PTX therapy was well tolerated in all patients. In particular, no PTX-related cardiac arrhythmia occurred. In contrast, PTX induced an evident decrease in the frequency of hemorrhagic symptoms in 9 of 11 patients with PTX-induced platelet normalization, whereas no clinical variation was seen in nonresponder patients.

Discussion

In accord with previous data [8], the present study showed that solid tumor-related DIC may be associated with abnormally elevated blood TNF levels, thereby suggesting a possible involvement of TNF in the onset of DIC. In addition, our results showed that there are two different groups of DIC patients, characterized by high or normal TNF values, respectively with low or normal-high fibrinogen levels. It has been shown that there are at least two subgroups of cancer-related DIC, with low or normal-high fibrinogen values [3,5]. The results of the present study suggest that the two DIC subgroups with different fibrinogen behavior reflect a different endogenous TNF secretion. In contrast, liver biochemistry did not seem to influence fibrinogen levels. The determination of blood TNF levels could thus contribute to the characterization of different pathogenetic mechanisms of DIC in human solid tumors, even though fibrinogen decline may simply depend on the evidence of liver damage. In any case, it could be that DIC is due at least in part to a TNF-induced direct endothelial damage in the presence of high TNF levels, whereas DIC with normal TNF values could reflect different pathogenetic mechanisms such as cancer cell production of procoagulant substances.

The possible involvement of TNF in the pathogenesis of cancer-related DIC is also suggested by the fact that a

PTX-induced decrease in TNF was associated with an increase in platelet count and clinical improvement in hemorrhagic symptoms. However, further studies to evaluate other important coagulatory parameters, including antithrombin III, are needed to better define any relation between TNF and biochemical alterations responsible for the onset of DIC. Moreover, owing to the different tumor histotypes, a larger number of patients is needed to confirm the negative correlation between TNF and fibrinogen production. In the same way, because of the possible influence of chemotherapy or supportive care, randomized studies are needed to confirm whether the increase in platelet count on PTX therapy is only due to PTX.

Caratterizzazione della coagulazione intravascolare disseminata dovuta a neoplasia in relazione ai livelli ematici di TNF: possibile ruolo terapeutico della pentossifillina.

Risultati sperimentali preliminari sembrano suggerire un possibile coinvolgimento del TNF nell'indurre il danno endoteliale associato alla DIC. Quest studi è stato c ndotto al fine di valutare la secrezion di TNF nella DIC in cors di neoplasia solida metastatica e di stabilire il possibile ruolo terapeutico della pentossifillina (PTX), rivelatasi in grado di ridurre i livelli di TNF. Lo studio è stato cond tt su 20 pazienti con tumore solido metastatico, che presentavano segni clinici e laboratoristici di DIC. La PTX è stata somministrata oralmente alla dose di 1200 mg/die per 28 giorni. Livelli anormalmente elevati di TNF erano presenti in 13/20 pazienti ed i livelli sierici medi di TNF riscontrati nei pazienti erano significativamente maggiori rispetto a quelli osservati in un gruppo di controllo di 50 soggetti sani. Le concentrazioni plasmatiche di fibrinogeno erano basse in 11 casi. I pazienti con bassi valori di fibrinogeno presentavano concentrazioni medie di TNF significativamente maggiori rispetto a quelli con valori normali o alti. La terapia con PTX determinava un calo significativo nei livelli medi di TNF ed un aumento significativo nel numero medio di piastrine, che si normalizzava in 11/20 pazienti. L'aumento delle piastrine in risposta alla PTX era più evidente nei pazienti con valori elevati di TNF prima del trattamento. Questo studio suggerirebbe l'esistenza di alti livelli ematici di TNF nella DIC in corso di neoplasia, in particolare in presenza di bassi valori di fibrinogeno. Inoltre questo studio suggerisce che la terapia con PTX può indurre un calo nei livelli di TNF nei pazienti affetti da DIC, evento questo che si associa ad un aumento nel numero delle piastrine.

References

1. Balkwill F., Burke F., Talbot D., Tavernier J., Osborne R., Naylor S., Durbin H., Fiers W.: Evidence for tumour necrosis factor/cachectin production in cancer. Lancet, 2: 1229-1231, 1987.
2. Bauer K.A., Ten Cate H., Barzegar S., Spriggs D.R., Sherman M.L., Rosenberg R.D.: Tumor necrosis factor infusions have a procoagulant effect on the hemostatic mechanisms of humans. Blood, 74: 165-172, 1989.
3. Bauer W.F.: Clinical aspects of disseminated intravascular coagulation. Semin. Thromb. Hemost., 15: 1-12, 1989.
4. Beutler B., Cerami A.: Cachectin: more than a tumor necrosis factor. N. Engl. J. Med., 316: 379-385, 1987.
5. Colman R.W., Rubin R.N.: Disseminated intravascular coagulation due to malignancy. Semin. Oncol., 17: 172-186, 1990.
6. Hawroth P.P., Stern D.M.: Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J. Exp. Med., 163: 740-743, 1986.
7. Strieter R.M., Remick D.G., Ward P.A., Spengler R.N., Lynch J.P., Larrick J., Kunkel S.L.: Cellular and molecular regulation of tumor necrosis factor-alpha production by pentoxifylline. Biochem. Biophys. Res. Commun., 155: 1230-1236, 1988.
8. Wada H., Ohiwa M., Kaneko T., Tamaki S., Tanigawa M., Takagi M., Mori Y., Shirakawa S.: Plasma level of tumor necrosis factor in disseminated intravascular coagulation. Am. J. Hematol., 37: 147-151, 1991.

STIC-ILL

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

JOURNAL OF INTERFERON AND CYTOKINE RESEARCH 15:819–825 (1995)
Mary Ann Liebert, Inc.

Signaling by E-selectin and ICAM-1 Induces Endothelial Tissue Factor Production via Autocrine Secretion of Platelet-Activating Factor and Tumor Necrosis Factor α

ESTHER SCHMID,[1] THOMAS H. MÜLLER,[1] RALPH-M. BUDZINSKI,[2] KLAUS BINDER,[1] and KLAUS PFIZENMAIER[3]

ABSTRACT

Based on previous studies showing adhesion molecule-dependent induction of tissue factor upon endothelium-lymphocyte interactions, we investigated whether E-selectin and ICAM-1 are linked to signaling pathways leading to tissue factor gene expression. Cellular interaction was mimicked by antibody cross-linking of E-selectin and ICAM-1 on the surface of human umbilical vein endothelial cells (HUVECs), resulting in induction of tissue factor mRNA and protein expression. Tissue factor production could be independently abolished by antibodies against TNF-α and by WEB 2086, a platelet-activating factor (PAF) receptor antagonist. Because WEB 2086 prevented the production and/or secretion of TNF-α by HUVECs, these results provide evidence for E-selectin- and ICAM-1-linked signal pathways leading to tissue factor synthesis in endothelial cells via an autocrine feedback loop involving PAF and TNF-α secretion.

INTRODUCTION

TISSUE FACTOR is regarded as the major trigger of the coagulation cascade both *in vitro* and *in vivo*.[1–4] Once tissue factor is expressed on the surface of endothelial cells, it forms a proteolytically active complex with activated factor VII (factor VIIa). This complex activates factor X and IX,[4–6] which ultimately leads to thrombin formation and fibrin generation. It is therefore assumed that activated endothelial cells play a major role in the induction of intravascular coagulation.

It was reported previously that leukocytes can induce endothelial tissue factor expression *in vitro*.[7–10] The underlying mechanism remained unclear. However, the binding of cell-associated ligands to endothelial adhesion molecules itself might be an important event, which may evoke intracellular signals, finally resulting in procoagulant activity. This idea is supported by the discovery that integrins as well as ICAM-1 are involved in signal transduction.[11–13] Indirect evidence for a role of adhesion molecules in tissue factor synthesis was obtained in our recent study,[14] in which we could block this process by separate or combined pretreatment of HUVECs with antibodies specific for ICAM-1 and E-selectin to prevent interaction with LFA1/sLex-positive CD4 T cells.

To investigate directly an active signaling function of adhesion molecules in tissue factor expression, second-passage human umbilical vein endothelial cells (HUVECs), either untreated or preactivated with interferon-γ (IFN-γ) to enhance E-selectin and ICAM-1 expression, were studied. It was previously shown that IFN-γ itself does not induce tissue factor expression on endothelial cells.[15,16] To mimic events solely caused by leukocyte adhesion in the absence of secreted leukocyte mediators, we cross-linked both adhesion molecules by treatment with anti-E-selectin and anti-ICAM-1, followed by incubation with antimouse IgG $F(ab)_2$. The influence of this receptor cross-linking on tissue factor mRNA and functionally active tissue factor production and the role of endogenous, endothelial cell-derived mediators in this process were studied.

[1]Department of Pharmacological Research, Dr. Karl Thomae GmbH, 88397 Biberach, Germany.
[2]Department of Biochemical Research, Dr. Karl Thomae GmbH, 88397 Biberach, Germany.
[3]Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany.

MATERIALS AND METHODS

Reagents

Recombinant human interferon-γ (Dr. K. Thomae GmbH, Biberach, Germany) was used at a final concentration of 50 ng/ml. Lipopolysaccharide (LPS from *Escherichia coli* 026:B6, Sigma, St. Louis, MO) was diluted to a final concentration of 1 μg/ml. WEB 2086 (Boehringer Ingelheim, Ingelheim, Germany), a direct antagonist of the platelet-activating factor receptor, was used at a final concentration of 4 mM. Platelet-activating factor (Boehringer Ingelheim, Ingelheim, Germany) was used at 10 μM.

Isolation and culture of cells

Endothelial cells from human umbilical cord veins were isolated according to the method of Jaffe et al.[17] and propagated in culture in medium 199 supplemented with CLEX (5% wt/vol; Interchem, Munich, Germany), heat-inactivated fetal calf serum (5% wt/vol), and human serum (5% wt/vol) until they reached confluence. The cells were detached from the culture dish by collagenase treatment (1 mg/ml in phosphate-buffered saline, PBS, 2 minutes, 37°C), washed in medium 199 plus supplementations, and then seeded on four-well plates (Nunclon; Nunc, Wiesbaden, Germany) coated with extracellular matrix derived from bovine corneal endothelial cells. For assays, cells of the second passage were grown to confluence for 2–3 days in medium 199 plus supplementations. Each well ($d = 16$ mm) contained approximately 1×10^5 endothelial cells.

Antibody staining and cytofluorometric analysis

Stimulated endothelial cells (IFN-γ, 50 ng/ml, 6 h) and control cells were washed and detached from the extracellular matrix by collagenase treatment as just described. Mouse monoclonal antibodies against tissue factor (American Diagnostica, Greenwich), E-selectin and ICAM-1 (Bender, Vienna), were used at a final concentration of 5 μg/ml each. Incubation was performed at 4°C for 45 minutes in PBS containing 0.2% bovine serum albumin (BSA). The cells were washed with PBS and BSA and stained with a fluorescein isothiocyanate (FITC)-conjugated $F(ab)_2$ goat antimouse IgG (5 μg/ml), for 45 minutes at 4°C. The cells were washed again and the pellet was resuspended in PBS and BSA. A control antibody of the same isotype (IgG_1, mouse, 5 μg/ml) was used to determine unspecific binding. Cytofluorometric analysis was performed with an Epics CS (Coulter, Hialeah, FL). Laser settings were 250 mW/25 A and a wavelength of 488 nm. The cytometer was calibrated using Dynabeads. Cells of interest (HUVECs) are shown after gating on forward and side scatter. Propidium iodide was used to determine viable cells.

Antibody cross-linking of E-selectin and ICAM-1

Monoclonal antibodies without preservatives were used. Anti-E-selectin (IgG_2, mouse; Bender, Vienna, Austria) and anti-ICAM-1 (murine, IgG_1; Bender, Vienna, Austria), as well as anti-CD34 (murine, IgG_1; Dianova, Hamburg, Germany) and anti-MHC class II (aHLA-DR, murine, IgG_2; Dianova, Hamburg, Germany), were added each at a final concentration of 50 μg/ml to the endothelial cells after IFN-γ treatment (50 ng/ml, 6 h) and washing with PBS. The incubations were performed at 4°C for 1 h. After washing again with PBS, the cross-linking antibodies were added. They consisted of $F(ab)_2$ fragments (goat antimouse IgG) at a final concentration of 500 μg/ml and were incubated with the endothelial cells for 1 h at 4°C, as described. After washing with PBS, the cells were further incubated for 12 h at 37°C.

Neutralizing antibodies and WEB 2086

All antibodies used were devoid of preservatives and added to the culture at a final concentration of 10 μg/ml. The antibodies, as well as WEB 2086, were incubated for the whole incubation period of 12 h at 37°C. Anti-TNF-α (murine, IgG_3) was purchased from Boehringer Mannheim, Germany. Anti-IL-2 (IgG_1 mouse) and anti-IL-1α (IgG_1, mouse) were obtained from Dianova (Hamburg, Germany).

Tissue factor activity

The surface procoagulant activity of the endothelial cell monolayer was measured in a two-stage thrombin formation assay in human whole blood. Freshly drawn citrated human blood (0.6 ml) was recalcified and immediately added to the endothelial cells. The four-well plate was gently shaken at 37°C. Samples (10 μl) were taken for 20 minutes at 1 minute intervals. The samples were diluted 1:400 in reaction buffer (Tris-HCl, 50 mM, NaCl, 100 mM, EDTA, 20 mM, and bovine serum albumin, 0.5 g/liter, pH 7.9) containing 200 μM chromogenic substrate S2238 (Kabi Vitrum, Sweden). The reaction mixture was kept on ice for the duration of the experiment, and then cellular components were removed by centrifugation. The chromogenic reaction was started by rapid warming up to 37°C. Absorption at 405 nm was measured at two time points (5 and 10 minutes after start of warming up), and optical density was determined. The amount of generated functionally active thrombin was calculated from a standard curve derived from purified human thrombin (kindly provided by Prof. Hemker, Maastricht, Netherlands). Next, a dose-response curve of recombinant human tissue factor (kindly provided by Prof. Konigsberg, Yale University, New Haven, CT) was prepared. We determined the time delay (minutes) until the rate of thrombin formation exceeded 30 nM as a parameter to convert the onset of thrombin formation to tissue factor concentration. The same method was used to determine the amount of tissue factor on the surface of endothelial cells.

Northern blot analysis

For northern blot analysis, endothelial cells were incubated with the cross-linking antibodies for only 2 h, instead of 12 h. Total cellular RNA was isolated by standard procedures. Electrophoresis of 10 μg RNA per slot was performed in a 1.2% agarose and 6.6% formaldehyde gel. The RNA was transferred to nitrocellulose (Hybond N membrane, Amersham) in 20-fold NaCl/citrate (150 mM NaCl and 15 mM sodium citrate, pH 7.0) and cross-linked by ultraviolet light with 1 J/cm^2. Human tissue factor cDNA (kindly provided by Prof. Konigsberg, Yale University) was labeled with a [^{32}P]dCTP (NEN) using a ready made DNA labeling kit (Pharmacia). Hybridization was per-

formed with 2×10^6 cpm/ml in 50% formamide, 5-fold Denhardt's, 5-fold solution A (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, and 1 mM EDTA) 0.5% sodium dodecyl sulfate (SDS), and 0.04 mg/ml of salmon sperm DNA for 18 h at 42°C. The blot was washed twice for 15 minutes in 2-fold solution A containing 0.1% SDS at 65°C, 30 minutes in 1-fold solution A plus 0.1% SDS at 65°C, and twice for 15 minutes in 0.1-fold solution A containing 0.1% SDS at room temperature. Hybridization with the constitutively expressed GAPDH was chosen for quantification. Autoradiography was done with intensifying screens at −70°C.

Measurements of tumor necrosis factor α (TNF-α) production

To determine the amount of TNF-α produced by the endothelial cells, the supernatants were tested by enzyme-linked immunosorbent assay (ELISA) using a human TNF-α ELISA kit (Boehringer Mannheim, Germany) according to the manufacturer's protocol.

RESULTS

Stimulation of HUVECs with IFN-γ

Interferon-γ-activated (50 ng/ml, 6 h) human umbilical vein endothelial cells show, compared with control cells, enhanced surface expression of E-selectin and ICAM-1.[14] In a typical experiment, the percentage of E-selectin-positive cells rose from 4.3 to 34%. ICAM-1-expressing cells increased from 77 to 90% after IFN-γ stimulation (Table 1). Furthermore, IFN-γ stimulation augmented the number of E-selectin and ICAM-1 molecules per cell, because the mean relative fluorescence intensity shifted from 11 to 24 and from 71 to 99 for E-selectin and ICAM-1, respectively.

Tissue factor transcripts in unstimulated or IFN-γ-activated HUVECs could not be detected by northern blot analysis (Fig. 1). The functional tissue factor activity of unstimulated and interferon-γ-stimulated HUVECs was low and did not accelerate thrombin formation in human whole blood (Fig. 2).

Effect of E-selectin and ICAM-1 receptor cross-linking on tissue factor induction

We used monoclonal antibodies against E-selectin and ICAM-1 for a cross-linking study. Very little tissue factor mRNA and only 7 ± 3 pg/ml of functionally active protein could be detected after cross-linking of resting cells with anti-E-selectin and anti-ICAM-1 antibodies (Figs. 1 and 2). Cross-linking of E-selectin and ICAM-1 on IFN-γ-preactivated cells, however, induced tissue factor mRNA (Fig. 1). The increased mRNA levels were followed by enhanced functional tissue factor activity (90 ± 8 pg, Fig. 2), which could be inhibited by anti–tissue factor antibodies (Fig. 2). Cross-linking of E-selectin alone and of ICAM-1 alone also induced functional tissue factor activity, but the response was lower and more variable, yielding approximately 60 ± 15 and 25 ± 10 pg/ml, respectively. Tissue factor induction by cross-linking was E-selectin and ICAM-1 specific, because cross-linking of other surface molecules (major histocompatibility complex II, MHC II, and CD34) on both IFN-γ-treated and unstimulated cells did not induce tissue factor mRNA or functional activity (Figs. 1 and 2). For comparison, stimulation of HUVECs with 1 μg/ml of lipopolysaccharide (LPS) induced approximately 180 ± 9 pg functionally active tissue factor.

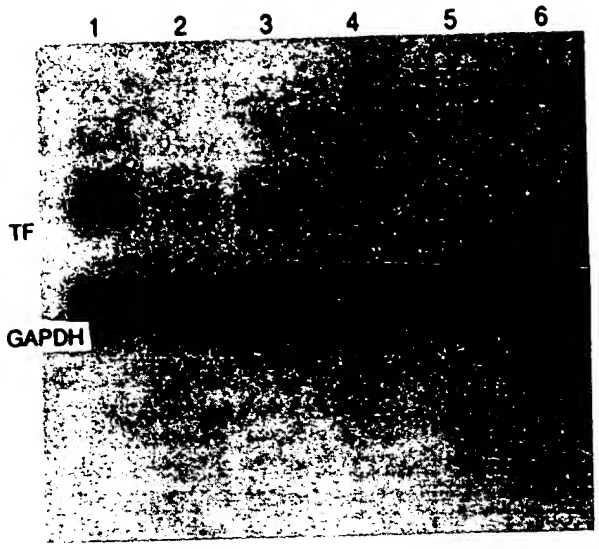


FIG. 1. Cross-linking of E-selectin and ICAM-1: northern blot analysis of tissue factor mRNA in HUVECs. Upper bands show 2.2 kb tissue factor mRNA, lower bands the 1.2 kb GAPDH mRNA. Lane 1, mRNA from IFN-γ-activated HUVECs, which were treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$. Lane 2, mRNA from resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$. Lane 3, mRNA from IFN-γ-activated HUVECs, treated with anti-MHC-II, anti-CD34, and antimouse IgG $F(ab)_2$. Lane 4, mRNA from resting HUVECs treated with anti-MHC-II, anti-CD34, and antimouse IgG $F(ab)_2$. Lane 5, transcripts from resting HUVECs. Lane 6, mRNA from IFN-γ-treated HUVECs. Data show one representative experiment of three.

TABLE 1. FACS ANALYSIS OF RESTING AND IFN-γ TREATED HUVECS[a]

	Control Ig	*Anti-E selectin*	*anti-ICAM-1*
Untreated HUVECs	3.2	4.3	77.0
IFN-γ-treated HUVECs	2.8	33.8	90.2

[a]Data are expressed as percentage of cells staining above background levels obtained with the secondary antibody alone. Shown is one representative experiment of three performed with similar results.

Inhibition of the platelet-activating factor receptor

Addition of the synthetic platelet-activating factor receptor antagonist WEB 2086 to the endothelial monolayer abolished the effect of cross-linking. Functional tissue factor activity was downregulated by WEB 2086 from 90 ± 8 pg to approximately 5 ± 3 pg (Fig. 3). LPS-induced tissue factor expression was not affected by WEB 2086 and remained at 190 ± 15 pg/ml of tissue factor. Stimulation with the platelet-activating factor (PAF)

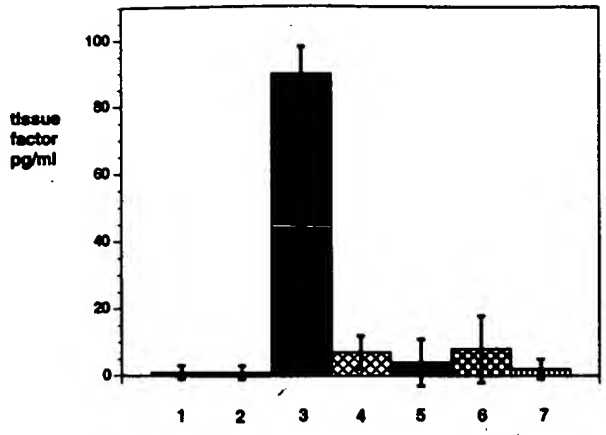


FIG. 2. Effect of cross-linking on functional tissue factor expression of HUVECs. Lane 1, resting HUVECs; lane 2, IFN-γ-treated HUVECs (50 ng/ml, 6 h); lane 3, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 4, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 5, IFN-γ-treated HUVECs incubated with antimouse IgG $F(ab)_2$; lane 6, IFN-γ-treated HUVECs cross-linked by anti-MHC-II, anti-CD34, and antimouse IgG $F(ab)_2$; lane 7, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$ followed by inhibition with anti–tissue factor antibodies (45 minutes, 50 μg/ml). Data show mean ± standard deviation (SD) of three experiments.

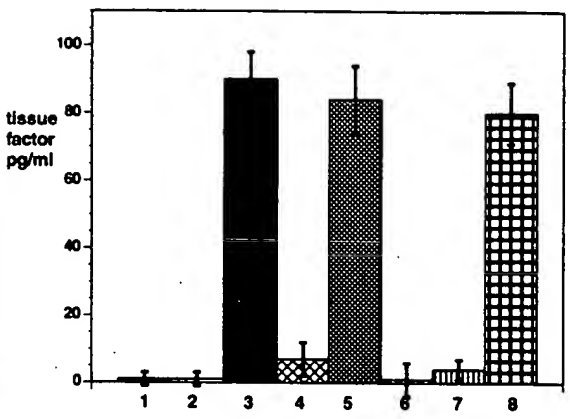


FIG. 3. Inhibition of cross-linking effect. Lane 1, resting HUVECs; lane 2, IFN-γ-treated HUVECs (50 ng/ml 6 h); lane 3, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 4, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 5, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$, followed by anti-IL-2 antibodies (37°C, 12 h); lane 6, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$, followed by anti-TNF-α antibodies, lane 7, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$, followed by WEB 2086 incubation (37°C, 12 h); 8, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$, followed by anti-IL-1α antibodies. Data show mean ± SD of three experiments.

itself, however, did not induce endothelial tissue factor expression, either on quiescent cells or on IFN-γ-prestimulated HUVECs.

Neutralization of soluble mediators

To investigate the role of soluble mediators in the induction of tissue factor, we separately employed neutralizing antibodies against TNF-α, interleukin-1α (IL-1α), and IL-2. Anti-TNF-α antibodies completely prevented tissue factor induction upon cross-linking of E-selectin and ICAM-1 (Fig. 3). In contrast, both anti-IL-2 and anti-IL-1α neutralizing antibodies had no effect on tissue factor induction (approximately 85 ± 10 and 80 ± 9 pg, respectively; Fig. 3).

Induction of endothelial TNF-α production by E-selectin and ICAM-1 cross-linking

TNF-α secretion was determined by enzyme-linked immunosorbent assay as described. Cross-linking of resting cells with anti-E-selectin and anti-ICAM-1 antibodies triggered the release of 180 ± 40 pg/ml of TNF-α, which was measured 5 h after cross-linking (Fig. 4), whereas cross-linking of E-selectin and ICAM-1 on the surface of IFN-γ-preactivated endothelial cells yielded significantly more TNF-α (490 ± 7 pg/ml, Fig. 4). The production of TNF-α by either resting or IFN-γ-prestimulated endothelial cells after cross-linking of E-selectin and ICAM-1 could be downregulated by the addition of the PAF receptor antagonist WEB 2086, which reduced the amount TNF-α produced to 130 ± 60 and 120 ± 28 pg/ml, respectively (Fig. 4). However, in the absence of cross-linking antibodies, stimulation of endothelial cells with either IFN-γ or PAF, or

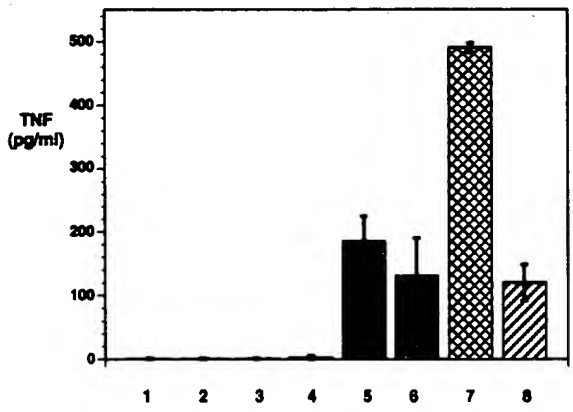


FIG. 4. ELISA measurements of endothelial TNF production. Lane 1, resting HUVECs; lane 2, IFN-γ-treated HUVECs; lane 3, PAF-treated HUVECs (10 μM); lane 4, IFN-γ + PAF-stimulated HUVECs; lane 5, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 6, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$ + WEB 2086; lane 7, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 8, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$ + WEB 2086. Data show mean ± SD of three experiments.

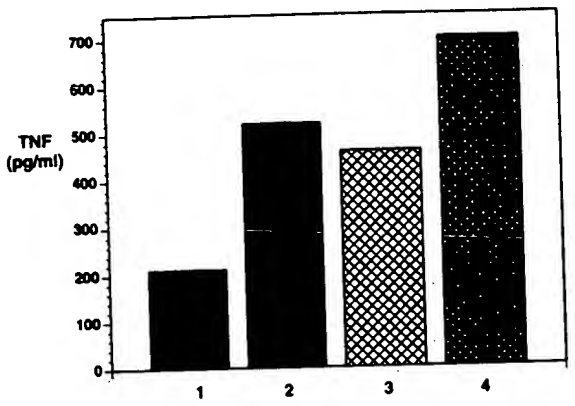


FIG. 5. ELISA measurements of endothelial TNF production. Lane 1, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 2, resting HUVECs treated with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$ + PAF (10 μM); lane 3, IFN-γ treated HUVECs cross-linked with anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$; lane 4, IFN-γ-treated HUVECs cross-linked by anti-E-selectin, anti-ICAM-1, and antimouse IgG $F(ab)_2$ + PAF (10 μM). Data show one representative experiment of three.

with both IFN-γ and PAF simultaneously, did not trigger the release of TNF-α (Fig. 4).

TNF-α production upon cross-linking of E-selectin and ICAM-1 in the presence of exogenous PAF

The release of TNF-α after cross-linking of E-selectin and ICAM-1 was downregulated by WEB 2086, thus indicating a costimulatory role of PAF. Because stimulation of endothelial cells with PAF alone did not trigger the release of TNF-α, we wanted to clarify the role of PAF with respect to TNF production. Addition of exogenous PAF to resting endothelial cells with cross-linked E-selectin and ICAM-1 led to enhanced levels of TNF in the supernatant, which rose from 210 to 520 pg/ml (Fig. 5). Addition of exogenous PAF to IFN-γ stimulated cells after cross-linking of E-selectin and ICAM-1 caused an increased in TNF production from 460 to 700 pg/ml (Fig. 5).

DISCUSSION

This work provides evidence that ligand binding to E-selectin and ICAM-1 activates a signaling pathway that induces tissue factor synthesis via PAF and TNF-α production.

Resting and IFN-γ-stimulated HUVECs had very little tissue factor mRNA and no procoagulant activity. However, addition of IFN-γ led to upregulation of ICAM-1 expression and also enhanced basal levels of E-selectin, as revealed from immunofluorescence flow cytometry. The upregulation of ICAM-1 by IFN-γ is in accordance with other reports,[18] and increased E-selectin expression upon IFN-γ stimulation was also observed previously,[19] although IFN-γ by itself does not induce expression of E-selectin. Rather, it has been shown that IFN-γ exerts modulating effects on E-selectin by enhancing and prolonging the expression of E-selectin on the surface of endothelial cells.[19] Accordingly, a similar mechanism can be assumed here, where a preexisting basal expression of E-selectin is further enhanced by IFN-γ. This reasoning is supported by the observation (E. Schmid, unpublished results) that normal endothelial cell cultures show variable and sometimes elevated levels of E-selectin, which may reflect cell culture-dependent differences in the preactivation/differentiation state of HUVECs. In any case, IFN-γ treatment did not induce tissue factor as determined by northern blot and functional analysis, which is in agreement with reports from other laboratories.[15,16]

ICAM-1 is known to be an important adhesion molecule for lymphocytes.[20] Previously, it was also shown that at least a subpopulation of lymphocytes is also able to bind to E-selectin.[21,22] So far, there has been no evidence to suggest that E-selectin and ICAM-1 are linked to a signaling pathway whose activation leads to the induction of tissue factor. Because we previously showed that CD4 T cell adhesion to endothelium via E-selectin/ICAM-1-induced tissue factor production,[14] we cross-linked E-selectin and ICAM-1 to mimic the natural receptor-ligand interaction and to demonstrate the importance of both adhesion molecules in tissue factor induction.

Using this stimulation protocol, we observed a strong induction in tissue factor mRNA levels and functional tissue factor. In contrast, antibodies against MHC class II and CD34 were not effective, indicating that tissue factor induction is E-selectin and ICAM-1 specific. Costimulation of both E-selectin and ICAM-1 was necessary to obtain an optimal tissue factor response. However, cross-linking of each molecule alone resulted in a small but significant tissue factor expression (data not shown). This is in accordance with our previous data showing that inhibition of CD4 T cell-HUVEC interaction via either E-selectin/sLex or ICAM-1/LFA-1 resulted in an overproportional reduction in tissue factor synthesis.[14] No tissue factor induction by E-selectin and ICAM-1 cross-linking occurred in resting HUVECs, most likely because of insufficient expression levels of E-selectin and ICAM-1 on resting HUVECs, thus resulting in inefficient cross-linking.

With respect to the signal pathways activated upon E-selectin and ICAM-1 cross-linking, we obtained evidence for the involvement of PAF/PAF receptor, because the PAF receptor antagonist WEB 2086 inhibited the effect of cross-linking. Likewise, we recently showed that WEB 2086 also inhibits tissue factor production upon lymphocyte adhesion to HUVECs.[14] Thus, upon cross-linking of E-selectin and ICAM-1 by antibodies or by natural, membrane-expressed ligands during cell contact, PAF is secreted and can function as a messenger acting via an autocrine, PAF receptor-mediated feedback loop. However, PAF itself was not sufficient to induce the tissue factor expression of HUVEC. Therefore, other autocrine mediators must be involved that are produced concomitantly or subsequent to the release of PAF by HUVECs.[23] IL-1α and TNF-α are likely candidates, because both can be produced by endothelial cells[24–28] and are known to induce endothelial tissue factor.[29–31] However, in our system, only the addition of anti-TNF-α antibodies prevented the induction of tissue factor, whereas anti-IL-1α antibodies were ineffective. Inhibition of TNF-α action was specific inasmuch as tissue factor induction by LPS was not affected by anti-TNF antibodies (data not shown).

The antibody inhibition data suggested that endothelial cells produce TNF-α upon cross-linking of E-selectin and ICAM-1. This could be verified by determination of TNF-α levels in culture supernatants. These data show that endothelial cells are indeed able to produce TNF-α upon appropriate activation of E-selectin and ICAM-1. Engagement of both E-selectin and ICAM-1 also induced PAF release, which apparently can act as a costimulator of autocrine TNF-α production via activation of its cognate receptor. Accordingly, at least two external signals appear to be required for autocrine TNF-α production of HUVECs: one is mediated by E-selectin and/or ICAM-1 cross-linking itself, and the second is induced upon PAF interaction with its membrane receptor. These data also suggest that E-selectin/ICAM-1 cross-linking, induced either experimentally by antibodies or under physiologic conditions by leukocyte adhesion,[14] can potentially activate several independent signal pathways. Our finding that adhesion to E-selectin and ICAM-1 leads to induction of signaling pathways is corroborated by recent evidence that ICAM-1 can activate tyrosine phosphorylation in brain endothelial cells[32] and is in full accordance with the current view of adhesion molecules as important signal transducers in several tissues.[33] Likewise, PAF also activates signal pathways, because stimulation of endothelial cells by PAF induces protein kinase C (PKC).[34] Transcription of the TNF-α gene is known to be PKC dependent.[35] TNF-α in turn can act via two types of receptors, both of which have been demonstrated to be expressed by endothelial cells.[36,37] Thus, autocrine TNF can bind to these receptors and trigger diverse signaling events, typically resulting in NF-κB activation.[38] Both PKC and NF-κB have been described as major regulators of tissue factor gene transcription.[39,40]

In conclusion, our study demonstrates that cross-linking of E-selectin and ICAM-1, either by natural ligand or antibody mediated, activates a signaling pathway in the endothelial cells that leads to the transcription of the tissue factor gene and to the expression of functionally active tissue factor protein. We propose that this is achieved via an apparently autocrine double-positive feedback loop with the following sequence: (1) engagement of E-selectin and ICAM-1, (2) activation of intracellular signal transduction pathways and release of PAF, (3) PAF-mediated enhancement of TNF-α production/secretion, and (4) TNF-α-mediated tissue factor transcription.

A pathophysiologic role of adhesion molecule-induced tissue factor expression can be deduced from a recent report of a concomittant induction of tissue factor and E-selectin on endothelial cells in an animal model of septic shock.[41] However, to what extent this mechanism of endothelial cell tissue factor production contributes to intravascular coagulation under conditions in which the circulation is flooded with TNF-α from other cellular sources remains unclear at present. Rather, we propose that this autocrine. TNF-α-dependent tissue factor production of endothelial cells could be of relevance for the development of local tissue damage in noninfectious, chronic degenerative diseases, such as atherosclerosis.

ACKNOWLEDGMENTS

The excellent technical assistance of Claudia Röpke and Bernhard Sperker is gratefully acknowledged.

REFERENCES

1. KIRCHHOFER, D., SAKARIASSEN, K., CLOZEL, M., TSCHOPP, T., HADVARY, P., NEMERSON, Y., and BAUMGARTNER, H. (1993). Relationship between tissue factor expression and deposition of fibrin, platelets and leukocytes on cultured endothelial cells under venous blood flow conditions. Blood **81,** 2050.
2. WARR, T., RAO, L., and RAPAPORT, S. (1990). Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of antitissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. Blood **75,** 1481.
3. TAYLOR, F.B.J., CHANG, A., RUF, W., MORRISSEY, J.H., HINSHAW, L., CATLETT, R., BLICK, K., and EDGINGTON, T.S. (1991). Letal *E. coli* septic shock is prevented by blocking tissue factor with monoclonal antibody. Circ. Shock **33,** 127.
4. BAUER, K.A., KASS, B.L., CATE, H.T., HAWIGER, J.J., and ROSENBERG, R.D. (1990). Factor IX is activated *in vivo* by the tissue factor mechanism. Blood **76,** 731.
5. NEMERSON, Y. (1966). The reaction between bovine brain tissue factor and factors VII and X. Biochemistry, **5,** 601.
6. KOMIYAMA, Y., PEDERSEN, A.H., and KISIEL, W. (1990). Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. Biochemistry **29,** 9418.
7. GALDAL, K.S. (1984). Thromboplastin synthesis in endothelial cells. Haemostasis **14,** 378.
8. LYBERG, T., GALDAL, K.S., EVENSEN, S.A., and PRYDZ, H. (1982). Thromboplastin (factor III) activity in human monocytes induced by immune complexes. Br. J. Haematol. **53,** 85.
9. CARLSEN, E., GAUDERNACK, G., FILION-MYKLEBUST, C., PETTERSEN, K.S., and PRYDZ, H. (1989). Allogeneic induction of thromboplastin synthesis in monocytes and endothelial cells. Biphasic effect of cyclosporin A. Clin. Exp. Immunol. **76,** 428.
10. BINDER, K., SCHMID, E., and MÜLLER, T.H. (1993). In: *DIC: Pathogenesis, Diagnosis and Therapy of Disseminated Intravascular Fibrin Formation.* Müller-Berghaus, G. (eds.) Amsterdam: Excerpta Medica, Elsevier Science Publishers B.V., p. 55.
11. WACHOLTZ, M.C., PATEL, S.S., and LIPSKY, P.E. (1989). Leukocyte function-associated antigen 1 is an activation molecule for human T cells. J. Exp. Med. **170,** 431.
12. ROTHLEIN, R., KISHIMOTO, T.K., and MAINOLFI, E. (1994). Cross-linking of ICAM-1 induces co-signalling of an oxidative burst from mononuclear leukocytes. J. Immunol. **152,** 2488.
13. VAN SEVENTER, G.A., SHIMIZU, Y., HORGAN, K.J., LUCE, G.E.G., WEBB, D., and SHAW, S. (1991). Remote T cell co-stimulation via LFA-1/ICAM-1 and CD2/LFA-3: demonstration with immobilized ligand/mAb and implication in monocyte-mediated co-stimulation. Eur. J. Immunol. **21,** 1711.
14. SCHMID, E., MÜLLER, T.H., BUDZINSKI, R.-M., PFIZENMAIER, K., and BINDER, K. (1995). Lymphocyte adhesion to human endothelial cells induces tissue factor expression via a juxtacrine pathway. Thromb. Haemost. **73,** 421.
15. ZUCKERMAN, S.H., and SURPRENANT, Y.M. (1989). Induction of endothelial cell/macrophage procoagulant activity: synergistic stimulation by gamma interferon and granulocyte-macrophage colony stimulating factor. Thromb. Haemost. **61,** 178.
16. CARLSEN, E., STINESSEN, M.B., and PRYDZ, H. (1987). Differential effect of α-interferon and α-interferon on thromboplastin response in monocytes and endothelial cells. Clin. Exp. Immunol. **70,** 471.
17. JAFFE, E.A., NACHMAN, R.L., BECKER, C.G., and MINICK, C.R. (1973). Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. **52,** 2745.
18. DUSTIN, M.L., ROTHLEIN, R., BHAN, A.K., DINARELLO, C.A., and SPRINGER, T.A. (1986). Induction of IL-1 and inter-

feron-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. **137,** 245.
19. LEEUWENBERG, J.F.M., VON ASMUTH, E.J.U., JEUNHOMME, T.M.A.A., and BUURMAN, W.A. (1990). IFN-γ regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells *in vitro*. J. Immunol. **145,** 2110.
20. DUSTIN, M.L., and SPRINGER, T.A. (1988). Lymphocyte function associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J. Cell. Biol. **107,** 321.
21. PICKER, L.J., KISHIMOTO, T.K., SMITH, C.W., WARNOCK, R.A., and BUTCHER, E.C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature **349,** 796.
22. SHIMIZU, Y., SHAW, S., GRABER, N., GOPAL, T.V., HORGAN, K.J., VAN SEVENTER, G.A., and NEWMAN, W. (1991). Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. Nature **349,** 799.
23. BUSSOLINO, F., BREVIARIO, F., TETTA, C., AGLIETTA, M., MANTOVANI, A., and DEJANA, E. (1986). Interleukin-1 stimulates platelet activating factor production in cultured human endothelial cells. J. Clin. Invest. **77,** 2027.
24. HANCOCK, W.W., SAYEGH, M.H., SABLINSKI, T., KUT, J.P., KUPIEC-WEGLINSKI, J.W., and MILFORD, E.L. (1992). Blocking of mononuclear cell accumulation, cytokine production, and endothelial activation within rat cardiac allografts by CD4 monoclonal antibody therapy. Transplantation **53,** 1276.
25. MARCEAU, F., GRASSI, J., FROBERT, Y., BERGERON, C., and POUBELLE, P.E. (1992). Effects of experimental conditions on the production of interleukin-1α and -1β by human endothelial cells cultured *in vitro*. Int. J. Immunopharmacol. **14,** 525.
26. NAGURA, H., and OHTANI, H. (1992). Expression of major histocompatibility class-II antigens by vascular endothelial cells leads to amplified immunoinflammatory processes. Acta Histochem. Cytochem. **25,** 653.
27. NAGANO, T., KITA, T., and TANAKA, N. (1992). The immunocytochemical localization of tumor necrosis factor and leukotriene in the rat liver after treatment with lipopolysaccharide. Int. J. Exp. Pathol. **73,** 675.
28. KAHALEH, M.B., and ZHOU, S. (1989). Induction of tumor necrosis factor TNF synthesis by endothelial cells upon exposure to R-TNF. Arthritis Rheuma. **32,** 124.
29. BEVILACQUA, M.P., POBER, J.S., and MAJEAN, G.R. (1984). Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular cells. J. Exp. Med. **160,** 618.
30. BEVILACQUA, M.P., POBER, J.S., and MAJEAN, G.R. (1986). Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc. Natl. Acad. Sci. USA **83,** 4533.
31. RYAN, J., BRETT, J., TIJBURG, P., BACH, R.R., KISIEL, W., and STERN, D. (1992). Tumor necrosis factor-induced endothelial tissue factor is associated with subendothelial matrix vesicles but is not expressed on the apical surface. Blood **80,** 966.
32. DURIEU-TRAUTMANN, O., CHAVEROT, N., CAZAUBON, S., STROSBERG, A.D., and COURAND, P.-O. (1994). Intercellular adhesion molecule-1 activation induces tyrosin phosphorylation of the cytoskeleton-associated protein cortactin brain microvessel endothelial cells. J. Biol. Chem. **269,** 12536.
33. CLARK, E.A., and BRUGGE, J.S. (1995). Integrins and signal pathways: the road taken. Science **268,** 233.
34. BUSSOLINO, F., SILVAGNO, F., GARBARINO, G., COSTA-MAGNA, C., SANARIO, F., ARESE, M., SOLDI, R., AGLIETTA, M., PESCARMONA, G., CAMUSSI, G., and BOSIA, A. (1994). Human endothelial cells are targets for platelet activating factor (PAF). Activation of alpha and beta protein kinase C iosozymes in endothelial cells stimulated by PAF. J. Biol. Chem. **269,** 2877.
35. CHUNG, I.Y., KWON, J., and BENVENISTE, E.N. (1992). Role of protein kinase C activity in tumor necrosis factor-alpha gene expression: involvement at the transcriptional level. J. Immunol. **149,** 3894.
36. MACKAY, F., LOETSCHER, H., STUEBER, D., GEHR, G., and LESSLAUER, W. (1993). Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J. Exp. Med. **177,** 1277.
37. SCHMID, E., BINDER, K., GRELL, M., SCHEURICH, P., and PFIZENMAIER, K. (1995). Both tumor necrosis factor receptors, TNFR60 and TNFR80, are involved in signalling endothelial tissue factor expression by juxtacrine TNF-α. Blood **86** (5) (in press).
38. HELLER, R.A., and KRÖNKE, M. (1994). Tumor necrosis factor receptor-mediated signaling pathways. J. Cell. Biol. **126,** 5.
39. HERBERT, J.-M., SAVI, P., LAPLACE, M.-C., DUMAS, A., and DOL, F. (1993). Chelerythrine, a selective protein kinase C inhibitor, counteracts pyrogen-induced expression of tissue factor without effect on thrombomodulin downregulation in endothelial cells. Thromb. Res. **71,** 487.
40. CROSSMAN, D.C., CARR, D.P., TUDDENHAM, E.G.D., PEARSON, J.D., and MCVEY, J.H. (1990). The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. J. Biol. Chem. **265,** 9782.
41. DRAKE, T.A., CHENG, J., CHANG, A., and TAYLOR, F.B.J. (1993). Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal *Escherichia coli* sepsis. Am. J. Pathol. **142,** 1458.

Address reprint requests to:
Prof. Dr. Klaus Pfizenmaier
Institute of Cell Biology and Immunology
University of Stuttgart
Allmandring 31
70569 Stuttgart, Germany

Received 16 May 1995/Accepted 1 June 1995

STIC-ILL RBI·L 2

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

0023-6837/88/5804-0365$02.00/0
LABORATORY INVESTIGATION

Vol. 58, No. 4, p. 365, 1988
Printed in U.S.A.

Biology of Disease

Acute Inflammation and Microthrombosis Induced by Endotoxin, Interleukin-1, and Tumor Necrosis Factor and their Implication in Gram-Negative Infection

MYRON I. CYBULSKY, M. K. WILLIAM CHAN, AND HENRY Z. MOVAT

Departments of Pathology and of Immunology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Introduction 365
Inflammation Induced by *E. coli* and Endotoxin 365
Morphologic observations 365
Quantitative studies 366
Mediation of Endotoxin-Induced Inflammation 367
Leukocyte chemoattractants 367
Cytokines: mediators of the acute phase response 367
Interleukin 1: mediator of endotoxin-induced neutrophil emigration 368
Endotoxin and cytokine-induced endothelial cell adhesiveness for leukocytes 369
Endotoxin-Induced Microvascular Injury and Thrombosis 370
Inflammation and Host Defense in Gram-Negative Infection 373

INTRODUCTION

Acute inflammation constitutes the body's principal mode of defense against infection and other harmful agents, and neutrophils are the primary effector cells in this process. When inflammation occurs in response to infection with pathogenic microorganisms, the damage that is often observed locally is a sacrifice aimed to prevent the spread of infectious agents throughout the body. Gram-negative microorganisms elicit a brisk inflammatory reaction which is largely induced by one of their cell wall constituents, endotoxin. The infiltrating neutrophils phagocytose and kill the bacteria. The inflammatory reaction is often associated with severe local microvascular injury and abscess formation. Besides eliciting inflammation, endotoxin can predispose the local microvasculature to thrombosis upon subsequent systemic endotoxemia or complement activation, as demonstrated by the local Shwartzman reaction. Both the inflammatory and the thrombotic phenomena induced by endotoxin are mediated by the local generation of cytokines.

In addition to local effects, endotoxin shed by Gram-negative bacteria can access the circulation resulting in profound systemic effects. Endotoxin has been suggested as the principal causative agent of Gram-negative septic shock and disseminated intravascular coagulation, which are associated with a high mortality. It is also capable of eliciting fever, various components of the acute phase reaction, and a prolonged and profound neutropenia.

In this review, we examine the inflammatory and thrombotic reactions elicited by a Gram-negative bacterium, *Escherichia coli* and by endotoxin, followed by an analysis of the *in vivo* and *in vitro* observations which implicate cytokines as the mediators of these phenomena. We then examine the type and the mechanisms of the resulting microvascular injury and deal briefly with the significance of inflammation in defense against Gram-negative microorganisms.

INFLAMMATION INDUCED BY *E. coli* AND ENDOTOXIN

MORPHOLOGIC OBSERVATIONS

By counting the number of neutrophils in the lymph draining an inflammatory lesion and examining the tissue histologically, injection of *E. coli* was found to elicit a very intense inflammatory reaction, resulting in abscess formation in 24-hour lesions in sheep (64). Similar observations, including the abscess formation, were made subsequently in rabbits, in which ultrastructurally phagocytosed bacteria were demonstrable in neutrophils, often undergoing lysis (69). More recently, the morphology of the inflammatory lesions induced by killed *E. coli* was examined again and the findings correlated with other parameters (for details *vide infra* under "Inflammation and Host Defense in Gram-Negative Infection") (27). When 20 sites were injected simultaneously with *E. coli* (6×10^8/site), a marked neutropenia developed and

very few neutrophils were detectable at the injected site, and many extracellular bacteria were detectable histologically. A reinjection of the same large number of bacteria after recovery from the neutropenia (during the neutrophilic phase), resulted in a marked infiltration of the dermis by neutrophils, with very few bacteria, mostly within the phagocytes.

The injection of killed *E. coli* or large doses of endotoxin is followed by severe microvascular injury (*vide infra*, "Endotoxin-Induced Microvascular Injury and Thrombosis"). However, the injection of a large number of live *E. coli* (2×10^{10}/site) or smaller numbers in neutropenic rabbits is associated with necrosis of the dermis at the injection site (18).

QUANTITATIVE STUDIES

Having been able to quantitate increase in vasopermeability (115) and changes in blood flow in inflammation (49), it had become pertinent to quantitate the emigration of neutrophil leukocytes and their accumulation in the lesions (55, 56). The first studies on quantitation and kinetics of the acute inflammatory reaction were done with killed *E. coli*. Neutrophils were isolated from the blood of rabbits, radiolabeled and reinfused intravenously. These studies ascertained that the emigration of neutrophils was transient. The maximal rate of emigration was between 2 to 3 hours and after 6 to 8 hours, the rate of emigration was less than 10% of maximal (Fig. 1*A*). With live *E. coli* (18, 86) and leukocyte chemoattractants (22) neutrophil emigration followed similar kinetics. When blood mononuclear leukocytes were radiolabeled (61), the maximal rate of monocyte accumulation into lesions induced by killed *E. coli* coincided with neutrophils, however monocytes continued to accumulate at approximately 25% of the maximal rate for at least 24 hours (Fig. 1*B*). The absolute number of neutrophils which accumulate in *E. coli* lesions during the first 6 hours greatly exceeds the number of monocytes, as is evident in histologic sections of early lesions. Neutrophils comprise over 30% of the rabbit's circulating leukocytes, whereas monocytes constitute less than 5%. Thus, the delivery of circulating neutrophils to the inflammatory site exceeds monocytes by at least 6-fold. Emigrated neutrophils die relatively early or leave the inflammatory site via lymphatics (64). Monocytes continue to accumulate and undergo transformation into macrophages, thus becoming the predominant cell in the late stages of *E. coli* inflammation.

The skin of rabbits was ideal to quantitate events in an inflammatory reaction because various doses or time points under study could be injected in triplicate or quadruplicate, including positive and negative controls. However, *E. coli* or endotoxin as inflammatory stimuli were suitable also for the study of inflammation in the lung (29, 30) and pleura (53, 54). Moreover, by a further purification of the ^{51}Cr-labeled blood neutrophils, and a comparison of the radiolabeled cells in the blood and in a pleural exudate, the specificity and precision of the quantitative procedure was markedly improved (27). This enabled the expression of the results as the number of infiltrating neutrophils/lesion in relation to the number of circulating neutrophils. The latter was demonstrated to influence the delivery of neutrophils at the inflammatory site. When a large dose of *E. coli* was administered, a profound and prolonged neutropenia developed and neutrophil emigration into the *E. coli*-injected intradermal sites was markedly attenuated (27).

In addition to leukocyte emigration, the injection of *E. coli* induced a transient hyperemia, enhanced vasopermeability, and hemorrhage (69). *E. coli* and endotoxin

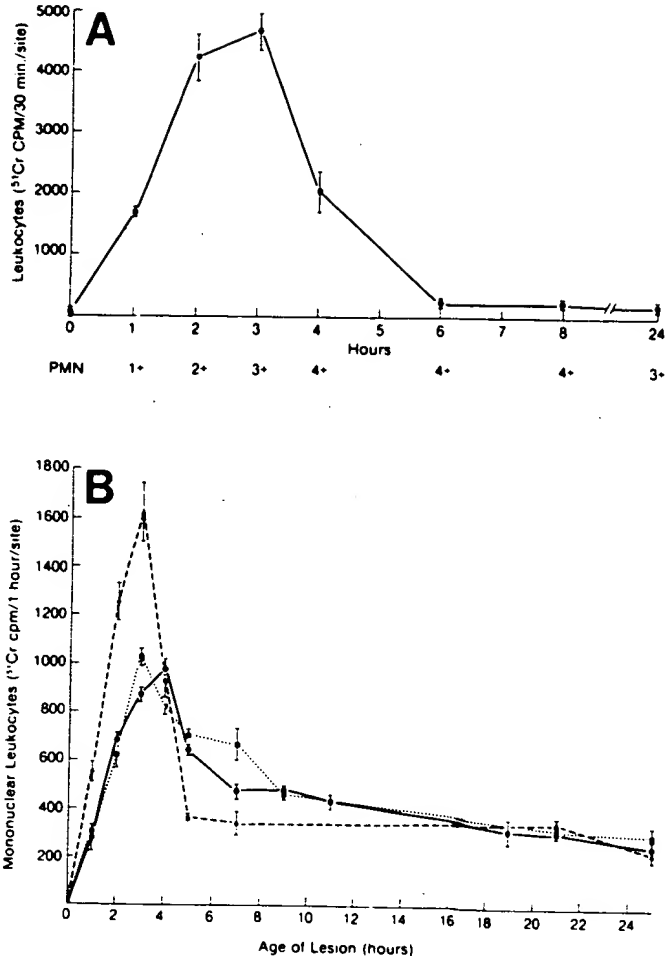


FIG. 1. Kinetics of leukocyte accumulation in rabbit skin. *A*, Rate of neutrophil leukocyte accumulation in inflammatory lesions induced by *E. coli*. Formalin-killed *E. coli* (5×10^8/site) were injected at varying times intradermally and autologous ^{51}Cr-labeled leukocytes were infused 30 minutes before sacrifice. Histologic examination ascertained that during peak emigration, over 90% of the infiltrating cell were neutrophil leukocytes (*PMNs*). Their number in hematoxylin and eosin-stained sections was scored semiquantitatively from 1 to 4+. This is one representative experiment of five. Points are means ± SEM of five replicate sites. Reproduced from Issekutz and Movat, Lab Invest 42:310, 1980. *B*, Mononuclear leukocyte accumulation in inflammatory lesions induced by *E. coli*. Killed *E. coli* (5×10^8/site) were injected intradermally at varying time intervals and ^{51}Cr-labeled mononuclear cells were injected intravenously 1 hour before sacrifice. The results of three experiments are shown. Points are mean ± SEM of triplicate sets. Saline-inoculated sites had 40 cpm, and this was subtracted from each point. By histology, the mononuclear cells were monocytes/macrophages. When ^{51}Cr-labeled lymphocytes were injected intravenously no accumulation was demonstrable. Reproduced from Issekutz *et al.*, Am J Pathol 103:47, 1981, Copyright by the American Association of Pathologists, Inc.

were the most frequent stimuli in inflammation associated with microhemorrhage (68) and microthrombosis (62, 87). Injected *E. coli* or endotoxin readily induce a hemorrhagic inflammatory reaction (69, 87), but this is seldom observed with staphylococcus-induced experimental inflammation (I. J. Cybulsky and H. Z. Movat, unpublished observations). Of all chemotaxins, only activated complement ($C5a_{des\ Arg}$) was capable of eliciting some hemorrhagic response (57, 58, 88). All the procedures mentioned above made use of radiolabeled cells and blood constituents for the quantitation of the inflammatory reaction.

Increase in vascular permeability and hemorrhage in inflammatory reactions induced by killed *E. coli* are neutrophil-dependent components of the reaction, since depletion of circulating neutrophils abrogates the development of vascular injury, quantitated with ^{125}I-albumin and ^{59}Fe-erythrocytes (70). This has been known for some time with respect to immune complex-induced inflammatory reactions associated with vascular injury (reviewed in Refs. 19, 81). The depletion of hemolytic complement and C5 markedly decreases neutrophil emigration, enhanced vasopermeability, and hemorrhage induced by the deposition of immune precipitates (24), however, it only partially inhibits the permeability change induced by *E. coli* and does not influence the neutrophil influx or hemorrhage (70). This suggested a mediating role for complement only in the immune complex-induced inflammatory reaction, and raised the question of a bacterium-derived substance in the mediation of *E. coli*-induced inflammation. Issekutz, Bhimji, and Bertolussi (52) demonstrated that killed *E. coli*-induced inflammation was diminished when the bacteria were treated with polymyxin B (which forms a complex with and inactivates endotoxin) or with anti-endotoxin antibody. Furthermore *E. coli* incubated in heat-inactivated plasma or buffer shed a substance into the supernatant, which induced neutrophil emigration when injected intradermally, but was not chemotactic *in vitro* (51). Polymyxin B or antibody to *O* or core glycolipid antigens diminished the potency of the supernatant (and of endotoxin) to induce neutrophil emigration. Issekutz and Bhimji (51) concluded that the material shed by the *E. coli* was endotoxin. Thus Gram-negative bacteria induce neutrophil emigration by releasing endotoxin which is not directly chemotactic or chemokinetic and at low doses exerts its effect *in vivo* independent of complement. During their growth phase *E. coli* secrete chemotactic formylated peptides (74) and potentially chemotactic lipids. Live *E. coli* induce also a neutrophil-independent tissue injury (18, 59), in which bacterial hemolysins play a role (59).

Attempts were also made to study the relationship between the movement of protein from the vasculature into the extracellular space and the clearance of this protein from the inflammatory site (induced by *E. coli* or other means). This was achieved by quantitating the increase in vascular permeability (^{131}I-albumin) and monitoring simultaneously the disappearance (clearance) of intradermally injected ^{125}I-albumin from inflammatory and control sites (47). It was observed that the removal rates of albumin injected into *E. coli*-induced inflammatory sites (10^2 to 10^6 *E. coli*/site) were not greater than those at sites injected with saline, despite 170 to 700% increases in vasopermeability observed in the inflammatory lesions. In fact, with high doses of *E. coli* (10^8/site) the mobilization of protein from the lesions was significantly reduced. In contrast with other inflammatory stimuli (bradykinin, heat injury) the clearance of extravascular protein from the lesions was enhanced over saline sites, implying a unique mechanism with *E. coli* (and endotoxin)-induced inflammatory edema (47). These observations may have relevance to the development of edema associated with Gram-negative septicemia and have led to studies on the role of lymphatic vessels in inflammatory edema (38, 63).

MEDIATION OF ENDOTOXIN-INDUCED INFLAMMATION

Gram-negative bacteria release (shed) endotoxins, which are lipopolysaccharide-protein complexes (40, 79, 120) and elicit a marked acute inflammatory reaction characterized by intense neutrophil emigration. The hypothesis that endotoxin-induced neutrophil emigration is a mediated process, is based on the knowledge that many of the *in vivo* biologic effects of endotoxin are mediated by host-derived mediators: leukocyte chemoattractants (C5a) and cytokines.

Leukocyte Chemoattractants

To date, substantial evidence has accumulated which suggest that leukocyte chemoattractants do not mediate endotoxin-induced neutrophil emigration. The depletion of complement with cobra venom factor had minimal effects on emigration induced by endotoxin (28) or *E. coli* (70), which indicates that C5a is not a relevant mediator. Picogram quantities of endotoxin are sufficient to elicit detectable neutrophil infiltration into the skin of rabbits and comparisons of molar potencies to leukocyte chemoattractants estimated that endotoxin was at least 1000-fold more potent (22, 26). Moreover, endotoxin did not induce tachyphylaxis to several leukocyte chemoattractants (20, 21, 26). The experiments of McComb, Cybulsky, and Movat (76) demonstrated that endotoxin induces neutrophil emigration by a different mechanism than leukocyte chemoattractants. The onset of neutrophil emigration following the injection of leukocyte chemoattractants was found to be more rapid and unlike endotoxin was not dependent on protein synthesis.

Cytokines: Mediators of the Acute Phase Response

The concept that cytokines are mediators of endotoxin-induced inflammation arose from observations made in conjunction with the acute phase response, which followed various forms of trauma and tissue injury associated with inflammation, but particularly the injection of endotoxin. The acute phase reaction includes fever, leukopenia followed by leukocytosis with neutrophilia, changes in certain heavy metals in the plasma, increase in acute phase proteins (e.g., C-reactive protein,

plasma proteinase inhibitors, transport proteins, fibrinogen) in plasma and their secretion in the liver (reviewed in Refs. 67, 81). Kampschmidt and co-workers described in the 60s and 70s a substance, "leukocytic endogenous mediator" which like the earlier described "endogenous pyrogen" and endotoxin, had the capacity to elicit the acute phase response (reviewed in Ref. 65). Both endogenous pyrogen and leukocytic endogenous mediator were shown to be leukocyte-derived, but the cell type was first in question, until monocytes/macrophages were demonstrated as the primary source of interleukin-1 (IL-1), endogenous pyrogen and lymphocyte-activating factor (2, 48, 121). However, more recently good evidence was again presented that neutrophils can synthesize IL-1 (122). Another important source of Il-1 with respect to inflammation is the endothelial cell (71, 92), and the smooth muscle cell (72). Lymphocyte-activating factor (in the thymocyte co-mitogenesis assay) was described in the 70s by immunologists and designated as IL-1 by a group of cellular immunologists (J Immunol 123:2928, 1979). Eventually endogenous pyrogen, leukocytic endogenous mediator and lymphocyte-activating factor were shown to be identical to IL-1, by comparing their biologic and biochemical properties (reviewed in Refs. 33, 34). Recently, tumor necrosis factor (TNF), a cytokine produced by monocytes-macrophages in response to endotoxin was found to possess many of the activities ascribed to IL-1 (6). In view of these observations, the roles of IL-1 and more recently of TNF in mediating neutrophil emigration induced by endotoxin were examined.

INTERLEUKIN 1: MEDIATOR OF ENDOTOXIN-INDUCED NEUTROPHIL EMIGRATION

Initial attention was focused on determining whether IL-1 could induce neutrophil emigration *in vivo*. Studies were carried out in which the activities in supernatants of cultured rabbit alevolar macrophages stimulated with opsonized zymosan or lipopolysaccharide, were assayed for neutrophil emigration-inducing activity and activity in the mouse thymocyte comitogenesis assay (25, 28). Fractions from a gel filtration column with low molecular weight showed activity in both assays, with peak activity in 14,000 to 16,000 molecular weight fractions, which corresponded to the MW of IL-1. High molecular weight fractions showed activity only in the neutrophil accumulation assay. Issekutz, Meygeri, and Issekutz (54) have reported similar activity in endotoxin-induced pleural exudates and supernatants of cultured pleural macrophages stimulated with endotoxin. Neutrophil emigration could also be induced with purified murine IL-1 (spirochete-stimulated macrophages, 5, 45), affinity-purified human monocyte-derived IL-1 (25, 26) and recombinant IL-1 preparations (28, 28a, 44, 96a). Observations with recombinant IL-1 are particularly important, since purified monocyte/macrophage-derived preparations may contain trace protein contaminants with biologic activities. Endotoxin contamination of IL-1 preparations has been ruled out by demonstrating stability of endotoxin but not of IL-1 to heating, and inhibition of endotoxin but not IL-1 activity with polymyxin B sulfate. Recently, TNF also has been found to induce neutrophil emigration (3, 28a, 31, 60, 85).

The analysis of the roles of IL-1 and TNF in endotoxin-induced neutrophil emigration is based on (a) comparisons of potencies, (b) kinetic profiles and (c) examination of cross tachyphylaxis. The potencies of both IL-1α and IL-1β approached that of endotoxin (27, 28a) (Fig. 2) and are similar to synthetic lipid A (28a). TNF on the other hand was found to be less potent than the IL-1 species (28, 85) (Fig. 2) and of comparable molar potency to the leukocyte chemoattractants. In earlier studies, leukocyte chemoattractants were found to be at least 1000-fold less potent than endotoxin (21, 26). These experiments suggest that in order to elicit an equivalent magnitude of neutrophil emigration, each molecule of endotoxin would have to generate 1 to 10 molecules of IL-1, 1000 to 10,000 molecules of TNF, or over 1000 molecules of a leukocyte chemoattractant. The latter possibility is not likely at least with regards to $C5a_{des\ Arg}$ or leukotriene B_4 since relatively high concentrations of endotoxin are required for the activation of complement (51) and endotoxin is not a potent stimulator of leukotriene B_4 production (13). The relative amounts of IL-1and TNF production by different cell types remains to be determined, however if both are produced, their effects on neutrophil emigration appear to be synergistic (60, 85) (Fig. 2).

The kinetic profile of neutrophil emigration induced by IL-1, TNF or endotoxin demonstrated a very low rate of emigration in the first 30 minutes (28, 28a). This may represent the time required by the endothelial cells to synthesize proteins adhesive for leukocytes (*vide infra*). After 30 minutes, emigration into sites injected with the cytokines increased dramatically, but remained low for a further 30 minutes into sites injected with endotoxin. These observations suggest that endotoxin may induce the synthesis of a cytokine mediator *in situ*, a process requiring approximately 30 minutes, followed by a further 30 minutes for the synthesis of adhesive proteins by the endothelium. In contrast to the cytokines or endotoxin, leukocyte chemoattractants elicit neutrophil emi-

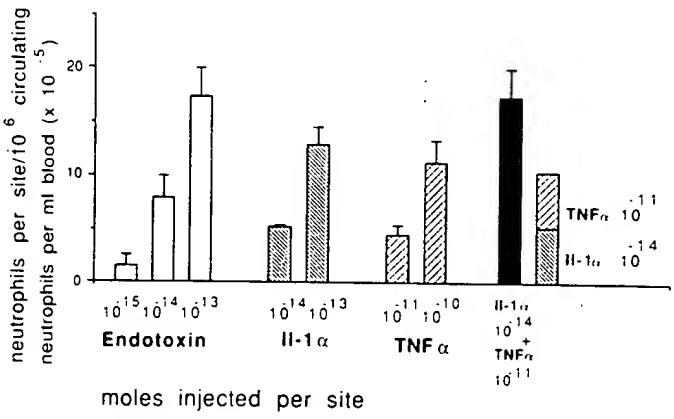


FIG. 2. Neutrophil emigration elicited with endotoxin, IL-1, TNFα or a mixture of the two cytokines, quantitated with ^{51}Cr-labeled neutrophils. The radiolabeled cells circulated for 2 hours after the intradermal injections and entered the inflammatory lesions during that time period. The dose response to two concentrations of IL-1α and TNFα are illustrated. When the lowest concentrations of the two cytokines were injected together, the neutrophil emigration was 69% greater than the additive response induced by each individual cytokine, implying a synergism between IL-1 and TNFα.

gration more rapidly, with significant emigration already detectable within the first 30 minutes after intradermal injections (76).

The term desensitization or tachyphylaxis at the inflammatory site was used in conjunction with reduced responsiveness to an inflammatory agent (20, 21). When dermal sites were reinjected with the same chemotaxin and the neutrophil emigration quantitated with ^{51}Cr-neutrophils after the reinjection (second injection), their number was decreased, compared with sites injected for the first time. Pertinent to this, was the observation that all inflammatory agents tested induced a transient emigration, which had ceased after the 4th hour (22). Thus, inflammatory lesions were initiated 8 hours before sacrifice and restimulated 6 hours later, followed immediately by the quantitation with the intravenously injected radiolabeled neutrophils. The tachyphylaxis was chemotaxin-specific, *i.e.*, sites initiated with *N*-formylmethionylleucylphenylalanine (FMLP) and restimulated with FMLP exhibited a reduced number of accumulated ^{51}Cr-neutrophils. In contrast, when FMLP-initiated sites were restimulated with platelet-activating factor, the same numbers of radiolabeled neutrophils accumulated as at sites injected for the first time with platelet-activating factor. The pathophysiologic role for tachyphylaxis may be to terminate inflammatory responses and to downregulate inflammation to chronically elevated (39) or physiologic (17) levels of cytokines. We utilized tachyphylaxis experiments to establish a potential role for cytokines generated *in situ* by endotoxin. Tachyphylaxis was noted at sites initiated and restimulated with endotoxin (21) and was observed also with natural IL-1 (26) and thereafter with recombinant IL-1 (27, 28a). Tachyphylaxis was dose-dependent; the higher the dose of the first stimulus, the fewer neutrophils accumulated after the reinjection.

Cross-tachyphylaxis was observed between IL-1α and IL-1β and between IL-1 and endotoxin (28a). When dermal sites were first stimulated with endotoxin and reinjected with IL-1, the response to IL-1 was reduced. Diminished neutrophil emigration was also observed when the order of endotoxin and IL-1 injections was reversed. This likely represents the desensitization of tissues to IL-1 generated in response to endotoxin, and implicates IL-1 as a potential mediator of endotoxin-induced neutrophil emigration. Cross-tachyphylaxis was not observed between TNF and endotoxin, suggesting that it is not a relevant mediator of neutrophil emigration.

In all these studies, the leukocytes infiltrating the dermis were almost exclusively neutrophils (Fig. 3).

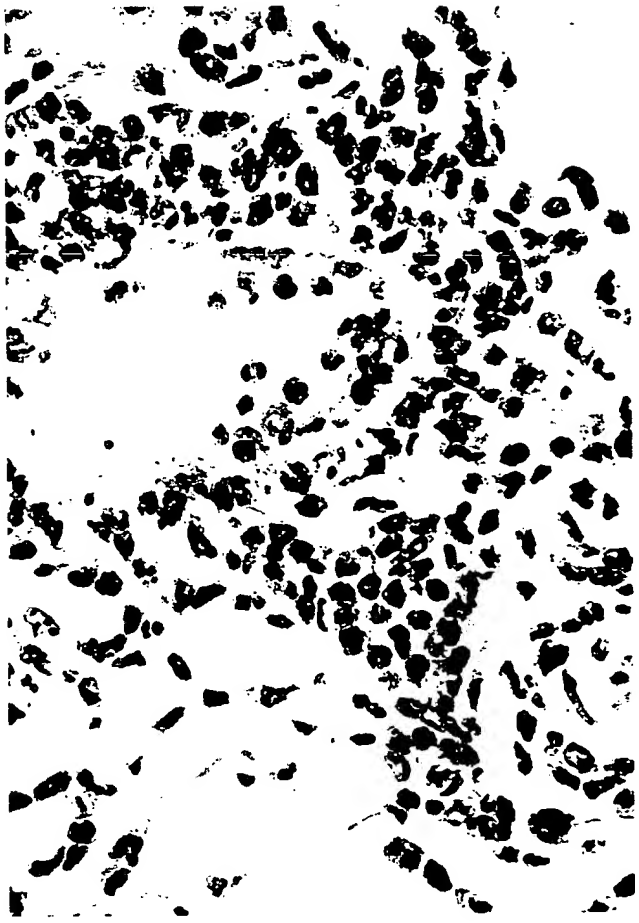


FIG. 3. Dermal inflammatory lesion (18 hours old) induced by the injection of 4 μg IL-1. Almost all the cells infiltrating the perivascular tissue are neutrophils. ×560.

ENDOTOXIN AND CYTOKINE-INDUCED ENDOTHELIAL CELL ADHESIVENESS FOR LEUKOCYTES

Leukocyte emigration into tissues through the walls of postcapillary venules and small veins can be divided into 3 steps: (a) adhesion of leukocytes to endothelial cells, (b) migration to intercellular junctions and diapedesis, (c) emigration through the endothelial basement membrane and vessel wall into extravascular tissues. An important regulatory step in emigration is the adhesion of leukocytes to endothelial cells, and is dependent both on endothelial and leukocyte mechanisms. Under certain physiologic conditions, leukocytes adhere weakly and transiently to the endothelium, which results in their rolling along venular walls: margination. Under pathologic conditions, particularly inflammation, margination is more pronounced until the leukocytes adhere strongly and eventually emigrate.

The advent of endothelial cell culture has led to *in vitro* studies of leukocyte-endothelial cell adhesion and elucidation of an endothelial cell-dependent adhesive mechanism. Both endotoxin and the cytokines IL-1, TNF and lymphotoxin induce endothelial cells to become adhesive for leukocytes by a mechanism dependent on protein synthesis (11, 37, 41, 103). Similarly, *in vivo* neutrophil emigration induced by endotoxin and IL-1 is dependent on protein synthesis, however this is not the case with leukocyte chemoattractants (76). A specific inducible endothelial cell surface protein designated by Gimbrone, Bevilacqua and colleagues as endothelial-leukocyte adhesion molecule 1 has been identified with two monoclonal antibodies: H4/8 (97) and H18/7 (10). It mediates, in part, neutrophil adherence to stimulated endothelial cells (10, 73). The induction of endothelial-leukocyte adhesion molecule 1 is transient, and endothelial cells become desensitized to restimulation with the

same cytokine or endotoxin, when the stimulus has been maintained in the culture medium (97, 98). *In vivo* endothelial-leukocyte adhesion molecule 1 has been identified in delayed type hypersensitivity reactions and in acute inflammatory conditions (23). The functional roles of other endothelial cell activation antigens (43) remain to be determined.

Leukocyte-dependent mechanisms for adhesion are predominantly mediated by a family of cell surface glycoproteins designated "LFA-1/Mac-1/p150,95" by Springer and co-workers (1,106) and CD11/CD18 by the Third International Workshop on Leukocyte Differentiation Antigens. These proteins are heterodimers with a distinct α-subunit (CD11a-c), noncovalently linked to a common β-subunit (CD18). Recurrent life-threatening bacterial infections developed by patients with hereditary deficiency of CD11/CD18 emphasizes its functional importance as reviewed by Anderson and Springer (1). Monoclonal antibodies to CD18 can block neutrophil adhesion to cytokine-stimulated endothelial cells (73, 99) and neutrophil emigration *in vivo* (1a).

ENDOTOXIN-INDUCED MICROVASCULAR INJURY AND THROMBOSIS

As described in the first section on quantitative studies, inflammatory reactions induced by *E. coli* or endotoxin are usually associated with severe injury, manifested by hemorrhage. It has been known for some time that endotoxin can elicit both local and systemic microvascular alterations, whose pathogenesis is not fully understood, and multiple mechanisms have been implicated (reviewed in Refs. 79, 80, 83).

Recent observations indicate that, like the neutrophil emigration, some of the microvascular alterations induced by endotoxin are mediated by IL-1 and TNF, and that neutrophils play a pivotal role in the development of the injury. As described in this review, neutrophil emigration can be induced by subnanogram quantities of endotoxin and both endotoxin and IL-1 are about 1000 times more potent in this respect than chemotaxins. For the elicitation of the classical local Shwartzman reaction, microgram quantities of endotoxin have to be injected locally ("preparative" injection), followed 18 to 24 hours later by an intravenous injection of endotoxin ("challenging" injection). The challenging injection of endotoxin could be substituted by a few procedures, but particularly by the intravascular activation of complement with immune precipitates, zymosan (82), or cobra venom factor (84). Rendering rabbits hypocomplementemic with cobra venom factor prevented the elicitation of a Shwartzman reaction when challenged with immune complexes or zymosan. However, when the intravenous challenge was endotoxin, the decomplementation induced only partial inhibition, implicating mainly complement-independent effects of the infused endotoxin (84). This substitution of endotoxin was more difficult with the preparative injection, until it was observed that with partially purified macrophage-derived IL-1 (presumably containing TNF) a Shwartzman-like reaction could be "prepared" (5, 84). However, when highly purified recombinant IL-1 was injected as a preparative dose, a thrombo-hemorrhagic Shwartzman-like reaction could only be elicited when recombinant TNF was injected simultaneously (84, 85). These lesions were quantitated with ^{59}Fe-erythrocytes (hemorrhage) and ^{111}In-platelets (thrombosis). Interestingly, sites prepared with endotoxin exhibited more thrombosis, and this was corroborated and extended morphologically. In addition to more numerous platelets, considerably more fibrin was seen in the lesions prepared with endotoxin than in those initiated with the cytokines. On the other hand, in the cytokine-induced lesions the hemorrhage was more intense.

These thrombo-hemorrhagic lesions which developed after the intravenous challenge, are superimposed on changes taking place locally after the preparative intradermal injections. In this respect, certain *in vitro* observations are important. These studies indicate that the endothelium plays an active role in coagulation, both in its promotion and inhibition (108). Both IL-1 and TNF can stimulate cultured endothelial cells to produce a tissue factor (thromboplastin)-like activity (8, 9, 93). By inhibiting fibrinolysis, IL-1 may play a role in the fate of a thrombus (12, 91). IL-1 and TNF added together to the endothelial monolayer were found to be additive with respect to induction of procoagulant activity, but *in vivo* with respect to the thrombo-hemorrhagic Shwartzman phenomenon they acted synergistically (84, 85). There is also *in vitro* evidence that endotoxin-stimulated endothelial cells synthesize IL-1 (71, 107). It is thus tempting to speculate that in addition to the inflammatory reaction induced by endotoxin via the cytokines at the injection sites, changes may be taking place at these sites which promote microthrombosis that develops after the intravenous challenge. It has been known for some time that intravenous injection, but particularly reinjection of endotoxin, leads to intravascular coagulation and consumption of clotting factors (80, 90). The reason why the microthrombi and hemorrhage were confined to the "prepared" site was the principal unanswered question, since the studies of Shwartzman (104). One decade ago innumerable intrinsic mediators were believed to be mediating events induced by endotoxin (79). Yet, none of these could fully account for its effects. Today we are still a long way from understanding the molecular events leading to a Shwartzman reaction, but the available evidence strongly suggests that IL-1 and TNF are the principal intrinsic mediators of this endotoxin-induced reaction.

Morphologic observations of the "prepared" sites immediately before intravenous challenge shed some light on the nature of these inflammatory lesions induced by endotoxin or IL-1 plus TNF, and thereby on the events which follow the intravenous challenge. Only when IL-1 and TNF were coinjected was the cellular infiltration comparable to that induced with endotoxin (31, 84, 85). Ultrastructurally, with endotoxin or IL-1 plus TNF, severe injury of venules and small veins was observed in conjunction with varying degrees of degenerative changes in neutrophils, both within and around the vessels. In these neutrophils granules had frequently fused with the cell membrane and were seen free in the lumen or abutting against endothelial cells. The latter exhibited focal or more extensive necrosis and frequently the basement membrane or remnants thereof was devoid of an endo-

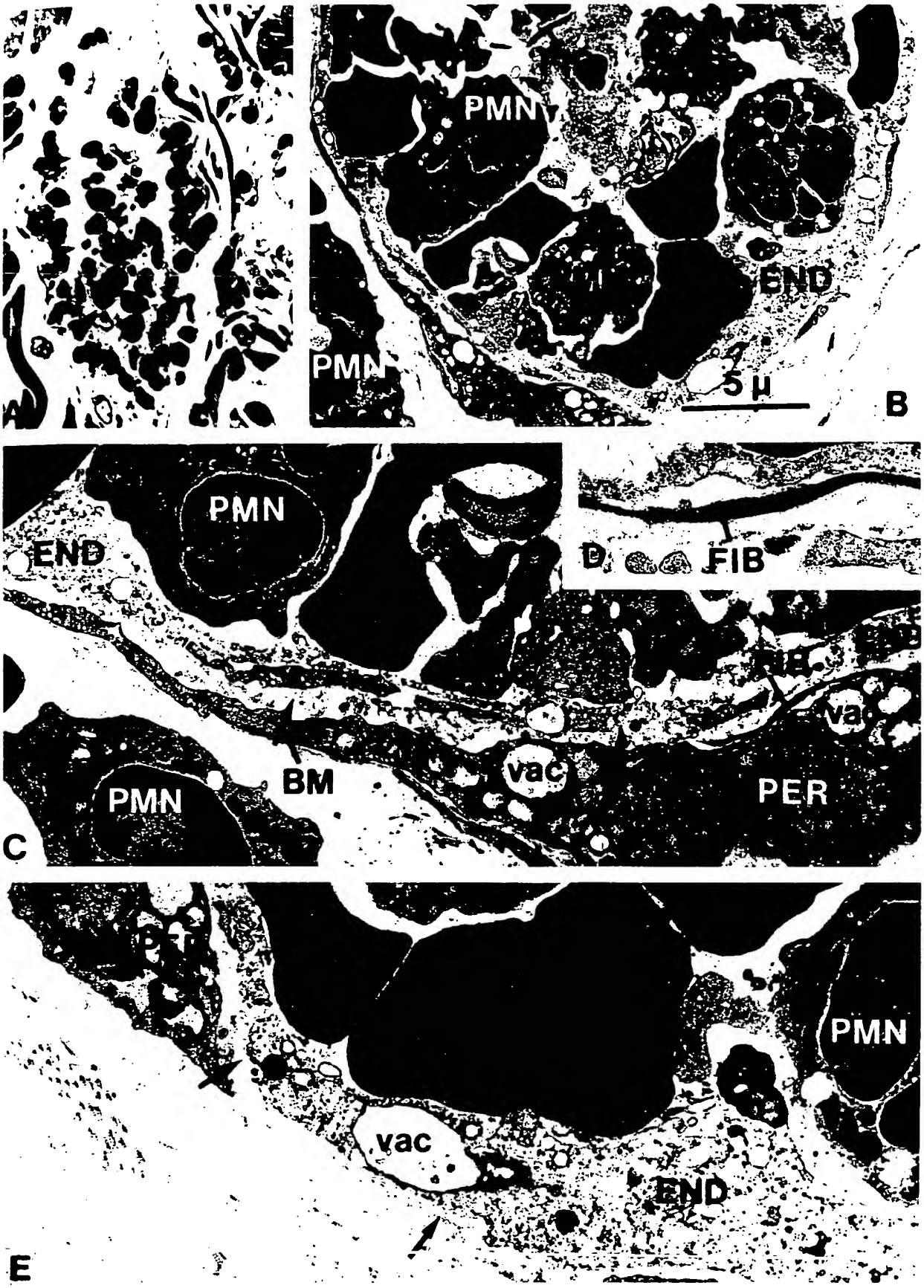


FIG. 4. Example of vessel wall injury. In Figure 4*A* (light microscopy) the endothelium is delineated by *arrowheads*. Neutrophils are present both in the lumen and perivascularly (inside and outside the *arrowheads*). About ½ the vessel is illustrated in the electron micrograph of *B*. Portions of the venular wall are reproduced in *C*, *D*, and *E*. The endothelial cells (*END*) are necrotic and the pericytes (*PER*) intact but vacuolated (*vac*). Some basement membrane (*BM*) surrounds pericytes, but the subendothelial basement membrane is fragmented or absent (*arrows*). Fibrin (*FIB*) is seen in the lumen of *B* and between endothelial cells and pericytes in *C* and *D* (*inset*). Figure 4*A*, ×560; *B*, × 4,125; *C*, ×12,000; *D*, ×33,900; *E*, ×12,000. Reproduced from Movat *et al.*, Am J Pathol 129:463, 1987, Copyright by the American Association of Pathologists, Inc.

thelial lining (Fig. 4). At times, the basement membrane was missing. Erythrocyte extravasation was associated with such changes (Fig. 5). This microvascular injury and the associated inflammatory hemorrhage gradually subsided. However, when a challenging intravenous injection of endotoxin, immune precipitates, zymosan, or cobra venom factor was administered at this time, microthrombi formed and occluded the vessels (Fig. 6). They consisted primarily of aggregated leukocytes, mostly neutrophils, but contained also aggregates of platelets and fibrin and were invariably accompanied by very extensive hemorrhage (85). The neutrophils in these thrombi also exhibited degenerative changes, but these were not seen in the monocytes and occasional lymphocytes. These events could be quantitated with radiolabeled cells and proteins. Early kinetic studies demonstrated that both thrombosis and hemorrhage developed rapidly and subsided 4 to 5 hours after the iv challenge (87).

The mediating role of IL-1 and TNF in the Shwartzman reaction is not fully understood as yet. Our studies on neutrophil emigration indicate that the cytokines, but particularly IL-1, are responsible for the neutrophil infiltration at the prepared site. The neutrophils are instrumental in the development of severe hemorrhage associated with the microvascular injury, because in neutropenic animals, a positive hemorrhagic reaction cannot be elicited (109) and because these cells showed signs of lysis and granule release. The only experimental model in which quantitatively and qualitatively, a similar vessel wall injury could be induced was that in which lysates of neutrophil lysosomes were injected intradermally (89).

There is no evidence in the literature that IL-1 can elicit hemorrhage, but TNF has been described to induce hemorrhagic necrosis of experimental tumors (101) and when massive doses (milligram quantities) were infused in rats, it elicited hemorrhage in several organs (114). Such quantities of TNF induce synthesis of IL-1 *in vitro* and *in vivo* (35), whereas stimulation with IL-1 induces production of IL-1, both *in vivo* and *in vitro* (36, 118). By itself even in very high doses, IL-1 was unable to "prepare" a site for the elicitation of a Shwartzman-like reaction; which was true also of TNF (84, 85). The term TNF is used interchangeably with TNFα and it is identical to cachectin, a substance implicated as the mediator of lethal endotoxin shock (6, 7, 114). By injecting merely microgram quantities, but both cytokines into rabbits, a synergism was demonstrated between IL-1 and TNF in

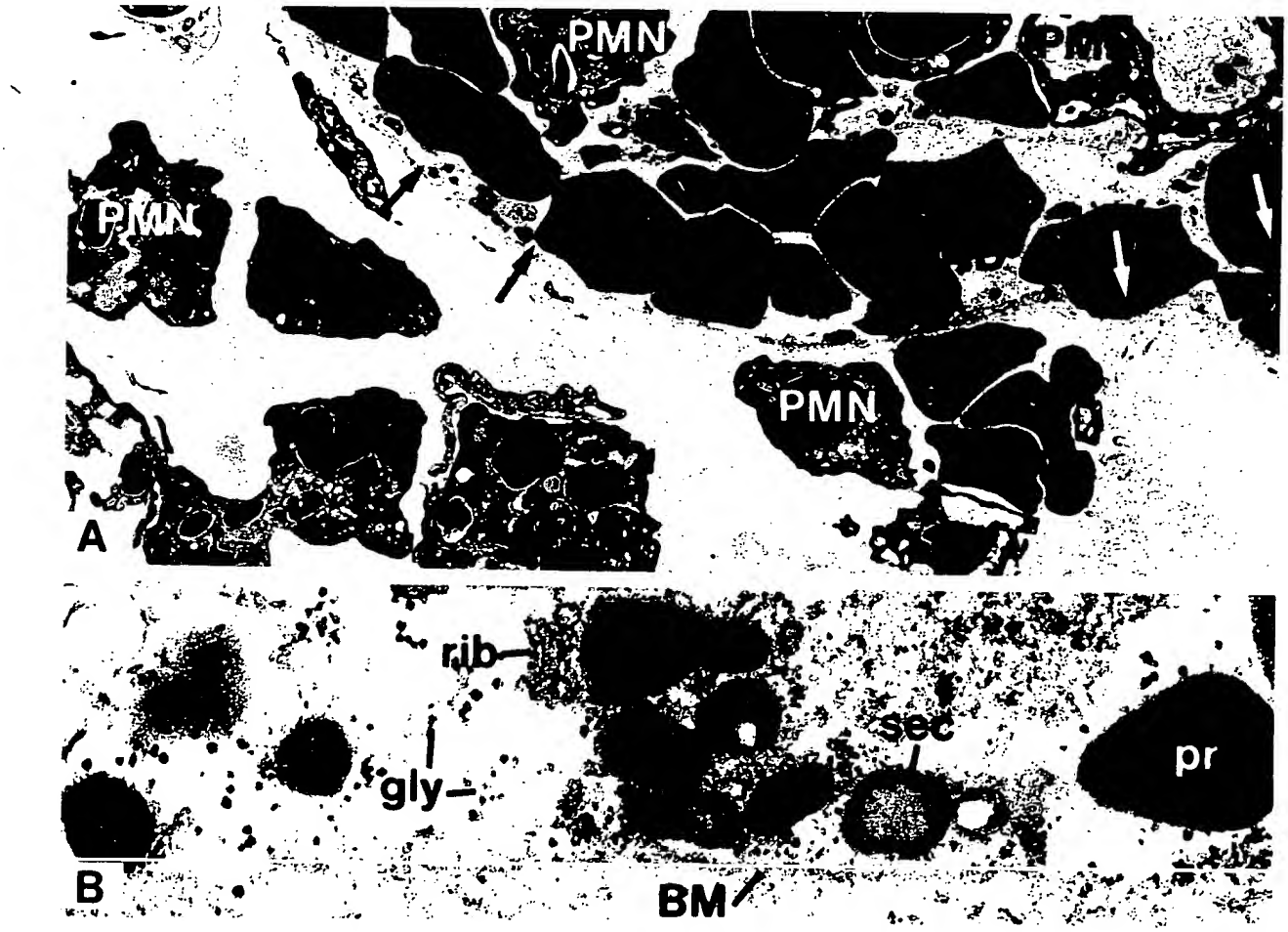


FIG. 5. Example of vessel wall injury. *A*, Most of the lining endothelium is missing. In the *right third* of the illustration, several erythrocytes (*RBC*) are in the perivascular tissue or in the process of crossing the injured vessel wall (*white arrows*). Eight neutrophils (*PMN*) are in the interstitium. *B*, The area between the two *black arrows* in *A*. There are free primary and secondary neutrophil granules (*pr, sec*), glycogen (*gly*), and ribosomes (*rib*) in the lumen, above the remnants of the basement membrane (*BM*). *A*, ×3,900; *B*, ×46,000. Reproduced from Movat *et al.*, Am J Pathol 129:463, 1987, Copyright by the American Association of Pathologists, Inc.

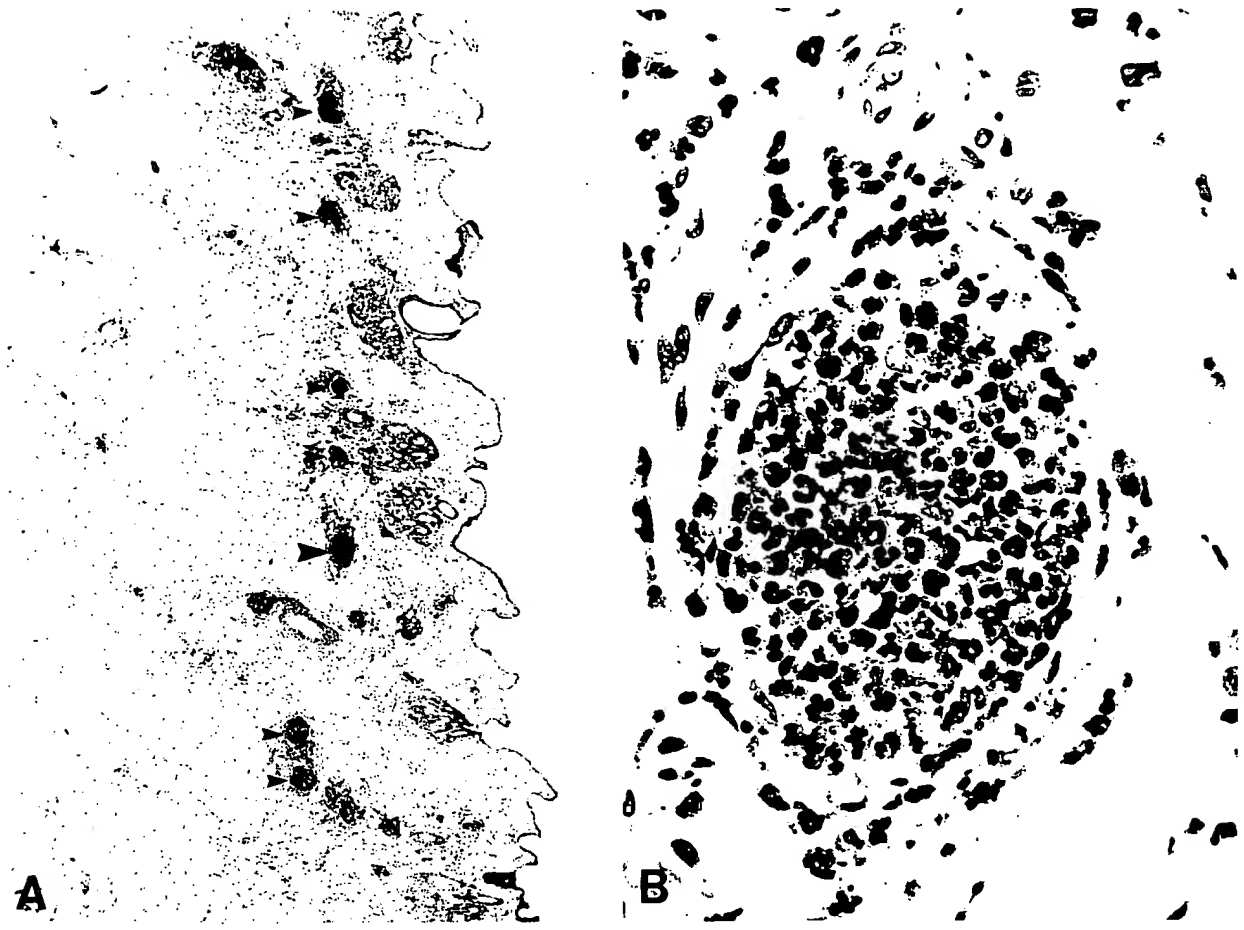


FIG. 6. Shwartzman-like reaction in rabbit skin prepared with intradermal injections of 0.5 μg of IL-1 and 1 μg of TNF. Eighteen hours after the intradermal injections, 10 μg/kg of endotoxin was administered intravenously and the rabbit sacrificed 15 minutes later. *A*, There are numerous venules occluded by microthrombi (small *arrowheads*). *B*, The microthrombi are made up mostly of neutrophils (vessel indicated by large *arrowhead* in *A*). Figure 6*A*, ×35; *B*, ×560.

the induction of a shock-like state with hemorrhagic pulmonary lesions (95). A leukocytopenia and thrombocytopenia were also observed in the last mentioned study and this observation may be important with respect to the intravenous challenging injection in the Shwartzman reaction. The rapid onset and prolonged leukopenia (predominantely neutropenia) observed after endotoxin infusion (29, 109, 110) or injection of *E. coli* (27) may well be mediated by IL-1, or by TNF or by IL-1 acting synergistically with TNF. In keeping with the observations of Okusawa *et al.* (95) on pulmonary changes, when ^{51}Cr-neutrophils were present in the circulation when endotoxin was injected, a large number of these cells were recovered in the lung (29). Furthermore, neutrophils were subsequently mobilized from the bone marrow and many of these ended up in the lungs (30). While the neutropenia after endotoxin or *E. coli* lasted (depending on the dose), from one to several hours, to be able to elicit a protracted shock-like state with leukopenia, Okusawa *et al.* (95) had to follow a bolus (1 μg) by a continuous infusion (5 ng/kg/min each of IL-1 and TNF). Interestingly, when considerably less IL-1 was administered to rats the cytokine was reported to induce only a neutrophilia, whereas TNF induced a neutropenia, followed by two peaks of neutrophilia (the second attributed to intrinsic IL-1 production) (117). There is even a possibility that the intravenous challenge with complement-activating agents may follow a pathway implicating IL-1 and TNF, since *in vitro* C5a and $C5a_{desArg}$ can stimulate blood mononuclear cells to produce IL-1 (94). Potential sources of IL-1 and TNF in the blood are primarily monocytes, and macrophages of the reticular activating system but there is also recent evidence for IL-1 production by neutrophils (111, 122).

INFLAMMATION AND HOST DEFENSE IN GRAM-NEGATIVE INFECTION

With the introduction of antibiotics, Gram-positive infections could be readily controlled, but Gram-negative bacteria became, during the 1950s, problems difficult to deal with, particularly in nosocomial infections (100). Antibiotics have only a limited effect on severe Gram-negative infections such as pneumonia, bacteremia, and septicemia (16, 77). Over a recent 5-year period (1977 to 1981), the mortality in 1,186 episodes of Gram-negative bacteremia was 36.3%, which was no improvement compared with 1924, *i.e.*, the presulfonamide and preantibiotic period (16). Whereas 70% of nosocomial lung infections are attributable to pseudomonas, and 33% to other Gram-negative bacteria, only 5% are caused by

Gram-positive infections (113). Another negative factor is the rapid development of resistance by the bacteria to an antibiotic in hospital-associated infections (102). Sepsis caused by Gram-negative bacteria, alone or in association with other aerobic and anaerobic bacteria, is the leading cause of the syndrome of multiple systems organ failure (4, 14) and is associated with a mortality of 50 to 80%. Should shock occur in association with sepsis, the mortality rate increases to 85 to 90% (105).

A number of bacterial properties contribute to their virulence, including leukocidins, capsules, adhesins, mobility, invasiveness, toxins, iron transport, and serum resistance (15). The diversity of the disease processes due to Gram-negative infection is attributed today mostly to a combination of virulence determinants associated with each bacterial species. For example, only a limited number of *E. coli* strains are usually associated with extraintestinal infections. Urinary tract infections are usually hemolytic (46) and are resistant to the bactericidal action of normal serum (112). One particular class of virulence-associated organelles are known as pili or fimbriae. These hair-like structures foster bacterial adherence to mucosal epithelium via specific receptor-ligand interactions (66). Piliation in *E. coli* permit efficient colonization of the gastrointestinal or genitourinary tracts by enteropathogenic and uropathogenic species respectively (78, 116).

In addition to the above variable factors determining virulence of Gram-negative bacteria, they produce various exotoxins, such as labile and stable enterotoxins (42, 50) and hemolysins (46, 119). However, these microorganisms have one disease-producing factor in common, endotoxin. Whereas the exotoxins are produced and released during bacterial growth, endotoxin is "released" by shedding also from dead bacteria. Release of endotoxin is marked during bacterial lysis (32). Endotoxin or lipopolysaccharide consists of lipid A, a core polysaccharide and the *O*-specific antigen and the biologic activity is known to reside in lipid A. The chemistry of endotoxin was elucidated primarily by Westphal and associates (for reviews see Refs. 40, 120). Endotoxin plays a particularly important role in severe and widespread infections, such as those associated with sepsis and septicemia, in which *E. coli* is the most frequent causative agent (75).

Killed *E. coli* have been shown to shed endotoxin into the medium in which they are suspended (51). Not surprisingly, such bacteria, particularly when deposited in large numbers (20 sites at 6×10^8 bacteria/site) into the dermis, will induce systemic effects attributed to endotoxin, such as fever and neutropenia, followed by neutrophilia (27). Both the magnitude and the duration of the neutropenia were dose-dependent. In these experiments, the number of circulating neutrophils was correlated with the infiltration at the site of *E. coli* injection. Compared with earlier observations where multiple injections of bacteria were administered over a 24-hour period before the quantitation with ^{51}Cr-neutrophils (69), when 20 simultaneous intradermal doses of killed *E. coli* were injected, very few neutrophils emigrated 0 to 4 hours postinjection (during the neutropenic phase). Instead, some influx of neutrophils occurred 6 to 10 hours postinjection (during the neutrophilic phase). When another set of 20 sites was injected 6 hours after the first set of 20 injections (during neutrophilic phase), the rabbits had become refractory to the development of a neutropenia, and a marked accumulation of neutrophils was quantitated in these lesions at 0 to 4 hours (27). The exact roles of various cytokines, and the direct or indirect effects of endotoxin on the levels of circulating neutrophils and their mobilization from the marrow pool remains to be determined.

Using killed *E. coli* has the advantage of fixed and reproducible stimuli. However, within certain dose limits, live *E. coli* elicit essentially similar inflammatory reactions as killed bacteria (86). We extended these studies with live *E. coli*, by comparing the magnitude and kinetics of ^{51}Cr-neutrophil accumulation with the level of circulating neutrophils, the increase in vasopermeability (^{125}I-albumin), and the recovery of bacterial colony-forming units (CFUs) in the dermal lesions of several groups of rabbits: normals, neutropenic (transient or persistent), immunized (active systemic or passive local) (18). Neutrophil emigration and enhanced vasopermeability peaked in 2 to 4-hour-old lesions (2×10^7 or 2×10^8 *E. coli*/site). However, it was essential to elicit at least a partial refractory state to neutropenia by injecting *E. coli* 24 hours before the experiment. This procedure induced a transient mild to moderate neutropenia and a subsequent neutrophilia. With high doses (2×10^{10} *E. coli*/lesion) a protracted neutropenia developed and no neutrophils were delivered to the stimulated sites. Bacterial pour plate colony counts from homogenates of intradermal sites injected with *E. coli*, indicated in normal rabbits early (1 to 2 hours) bacterial multiplication, followed by diminished CFUs at later time points. The decrease in CFUs coincided with the peak emigration of neutrophils into the dermal sites and light and electron microscopy demonstrated phagocytosis of the bacteria by the neutrophils. Animals rendered neutropenic for prolonged periods with nitrogen mustard showed a progressive increase in the numbers of *E. coli* within the intradermal sites, which by 48 hours increased several hundred times over controls. Compared with normals, fewer neutrophils accumulated and there was less plasma exudation. A delayed inflammatory reaction was observed during recovery from neutropenia, as noted already by Issekutz *et al.* (59). As in the lesions with 2×10^{10} *E. coli*/site, in the neutropenic rabbits there was severe dermal necrosis at sites injected with 2×10^7 *E. coli*/site. Animals which were injected with *E. coli* at the time when their circulating neutrophils were recovering from nitrogen mustard treatment, showed only an early (1 to 4 hour) increase in numbers of CFUs, followed by bacterial clearance. Two interesting observations were made in actively immunized animals: no neutropenia developed after the first intradermal injections of *E. coli*, and considerably fewer neutrophils accumulated in the dermis, but interestingly, these were able to cope with bacterial elimination in the same manner as normal controls. Fewer neutrophils accumulated, also with local passive immunization, i.e., when anti-*E. coli* antiserum was deposited at the injection site, but without the capacity to prevent the development of neutropenia.

It was concluded that (a) Neutrophil leukocytes play

a pivotal role in controlling the growth and spread of *E. coli*. (b) In the absence of neutrophils, the bacterial replication is unchecked. (c) In actively immunized rabbits fewer neutrophils can control the infection, (implying that (i) the cellular defense operates more efficiently and (ii) specific antibodies can assist and expedite the clearance of bacteria). (d) Passive transfer of antiserum locally also potentiates the clearance of microorganisms from the site of infection/inflammation. (e) In actively immunized animals no transient neutropenia develops in the circulation.

We propose that cytokines and in particular IL-1 are the principal mediators responsible for mobilizing neutrophils to the inflammatory site where endotoxin is released from Gram-negative bacteria. In immunized animals, fewer neutrophils infiltrated the inflammatory lesions, presumably due to neutralization of endotoxin in the outer wall of the bacteria, leading to less cytokine production, and hence less emigration. Diminished emigration was observed when *E. coli* or endotoxin were treated with antibody or polymyxin B (52). Likewise, when we injected *E. coli* for the first time into actively immunized rabbits, no transient neutropenia developed, probably because the locally liberated endotoxin was bound and this prevented stimulation of IL-1 and TNF synthesis and release. In addition to opsonization, antibody may have another effect in Gram-negative infection, *i.e.*, prevention of a decrease in circulating neutrophils and hence assurance of a constant delivery of neutrophils to the infected site.

Figure 7 illustrates our concept of the inflammatory events which follows the entry of Gram-negative bacteria into tissues. This diagrammatic presentation disregards exotoxins and emphasizes endotoxin and its effects: generation of host-derived inflammatory mediators and their action locally and systemically.

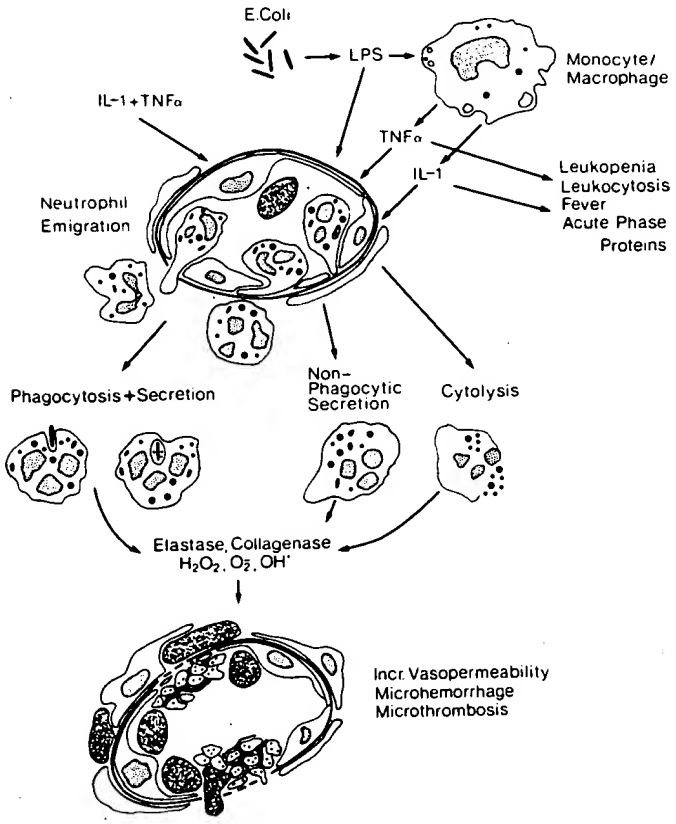


FIG. 7. Diagrammatic representation of events which follow entry of *E. coli* into tissues. The bacteria shed endotoxin (*LPS*) which stimulates monocytes and macrophages (locally and systemically) to synthesize and release IL-1 and TNFα. By acting on the circulating, marginal, and bone marrow pools of neutrophils and their precursors, the cytokines induce neutropenia, followed by neutrophilia (also lymphopenia). These events influence the delivery of neutrophils at the inflammatory site. Other systemic effect of the cytokines are elicitation of fever, acute phase proteins and other components of the acute phase reaction. Through action on the endothelium, IL-1 and TNFα induce neutrophil emigration and accumulation at the site of bacterial multiplication, representing the most important line of defense. IL-1 and TNFα of other sources could also induce neutrophil influx. *In vitro* studies indicate that endotoxin could act directly on the endothelium to induce synthesis and release of IL-1. The emigrated neutrophils release lysosomal enzymes and oxygen radicals by cytolysis, phagocytosis of the bacteria, and secretion and perhaps by nonphagocytic secretion. The severely injured microvessels exhibit increase in vasopermeability, hemorrhage, and thrombosis.

This work was supported by grants from the Medical Research Council of Canada (MT-1251) and the Heart and Stroke Foundation of Ontario (T082).

Dr. Cybulsky is the recipient of a Medical Research Council of Canada Research Fellowship; present address: Department of Pathology, Vascular Research Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115.

Dr. Movat is a Career Investigator of the Medical Research Council of Canada.

Address reprint requests to: Henry Z. Movat. M.D., Department of Pathology, University of Toronto, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8 Canada.

REFERENCES

1. Anderson DC, Springer TA: Leukocyte adhesion deficiency: an inherited defect in the Mac-1 LFA-1, and p150, 95 glycoprotein. Annu Rev Med 38:175, 1987
1a. Arfors K-E, Lundberg C, Lindbom L, Lundberg K, Beatty PG, Harlan JM: A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage *in vivo*. Blood 69:338, 1987
2. Atkins E, Bodel P, Francis L: Release of an endogenous pyrogen *in vitro* from rabbit mononuclear cells. J Exp Med 126:357, 1967
3. Averbrook B, Ulitch T, Jeffes E, Yamato R, Chow G, Masunaka I, Granger G: Human alpha lymphotoxin and TNF induce different types of inflammatory responses in normal tissues (abstr). Fed Proc 46:562, 1987
4. Baue AE, Chaudry IH: Prevention of multiple systems failure. Surg Clin North Am 60:1167, 1980
5. Beck C, Habict GS, Benach JL, Miller F: Interleukin 1: A common endogenous mediator of inflammation and the local Shwartzman reaction. J Immunol 136:3025, 1986
6. Beutler B, Cerami AC: Cachectin. More than a tumor necrosis factor. N Engl J Med 3156:379, 1987
7. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effects of endotoxin. Science 229:869, 1985
8. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA Jr: Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin. Proc Natl Acad Sci USA 83:4533, 1986
9. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone, MA Jr: Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cell. J Exp Med 160:681, 1984
10. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr: Identification of an inducible endothelial-leukocyte adhesion molecule. PNAS 84:9238, 1987
11. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone

MA Jr: Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. J Clin Invest 76:2003, 1985
12. Bevilacqua MP, Scheef RR, Gimbrone MA Jr, Loskutoff DJ: Regulation of the fibrinolytic system of cultured vascular endothelium by interleukin 1. J Clin Invest 78:587, 1986
13. Bonny RJ, Humes JL: Physiological and pharmacological regulation of prostaglandin and leukotriene production by macrophages. J Leukocyte Biol 35:1, 1984
14. Borzotta AP, Polk HC Jr: Multiple systems organ failure. Surg Clin North Am 63:315, 1983
15. Brubaker RR: Mechanisms of bacterial virulence. Annu Rev Microbiol 39:21, 1985
16. Bryan CS, Reynolds KL, Brenner ER: Analysis of 1,186 episodes of gram-negative bacteremia in non-university hospitals: the effect of antimicrobial therapy. Rev Infect Dis 5:629, 1983
17. Cannon JG, Dinarello CA: Increased plasma interleukin-1 activity in women after ovulation. Science 227:1247, 1985
18. Chan W, Movat HZ: Microbicidal role of inflammation in Gram negative infection (abstr). Fed Proc, in press 1988
19. Cochrane CG, Janoff A: The Arthus reaction: a model of neutrophil and complement-mediated injury. In The Inflammatory Process, Vol 3, edited by Zweifach BS, Grant L, McCluskey RT, p 85. New York, Academic Press, 1974
20. Colditz IG, Movat HZ: Chemotactic factor-specific desensitization of skin to infiltration by polymorphonuclear leukocytes. Immunol Lett 8:83, 1984
21. Colditz IG, Movat HA: Desensitization of acute inflammatory lesions to chemotaxins and endotoxin. J Immunol 133:2163, 1984
22. Colditz IG, Movat HZ: Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. J Immunol 133:2169, 1984
23. Cotran RS, Gimbrone MA Jr, Bevilacqua MP, Mendrick DL, Pober JS: Induction and detection of a human endothelial activation antigen *in vivo*. J Exp Med 164:661, 1986
24. Crawford JP, Movat HZ, Minta JO, Opas M: Acute inflammation induced by immune complexes in the microcirculation. Exp Mol Pathol 42:175, 1985
25. Cybulsky MI, Colditz IG, Movat HZ: Interleukin 1 activity in the local recruitment of PMNs. Its potential role in endotoxin-induced acute inflammation (abstr). Fed Proc 44:1260, 1985
26. Cybulsky MI, Colditz IG, Movat HZ: The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. Am J Pathol 124:6, 1986
27. Cybulsky MI, Cybulsky IJ, Movat HZ: Neutropenic responses to intradermal injections of *Escherichia coli*. Effects on the kinetics of polymorphonuclear leukocyte emigration. Am J Pathol 124:1, 1986
28. Cybulsky MI, McComb DJ, Dinarello CA, Movat HZ: Mediation by interleukin-1 of neutrophil leukocyte emigration induced by endotoxin. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 38. Basel, S Karger AG, 1987
28a. Cybulsky MI, McComb DJ, Movat HZ: Neutrophil leukocyte emigration induced by endotoxin: mediator roles of IL-1 and tumor necrosis factor α. J Immunol, in press 1988
29. Cybulsky MI, Movat HZ: Experimental bacterial pneumonia in rabbits: polymorphonucler leukocyte margination and sequestration in rabbit lungs and quantitation and kinetics of ^{51}Cr-labelled polymorphonuclear leukocytes in *E. coli*-induced lung lesions. Exp Lung Res 4:47, 1982
30. Cybulsky MI, Movat HZ: Application of ^{51}Cr-labelled PMN leukocytes in quantitating PMN kinetics in systemic and local inflammatory mediated processes. Effects of endotoxin, complement and interleukin 1. Surv Synth Pathol Res 1:208, 1983
31. Cybulsky MI, Movat HZ, Dinarello CA: Role of interleukin 1 and tumor necrosis factor α in acute inflammation. Ann Inst Pasteur, (Paris), 138:505, 1987
32. De Voe IW: Egestion of degraded meningococci by polymorphonuclear leukocytes. J Bacteriol 125:258, 1976
33. Dinarello CA: Interleukin-1. Rev Infect Dis 6:51, 1984
34. Dinarello CA: Interleukin-1. Amino acid sequence, multiple biological activities and comparison with tumor necrosis factor (cachectin). Year Immunol 2:68, 1986
35. Dinarello CA, Cannon JC, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA Jr, O'Connor JV: Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 163:1433, 1986
36. Dinarello CA, Ikejina T, Warner SJC, Orecole SF, Lonnemann G, Cannon JG, Libby P: Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits *in vivo* in human mononuclear cells *in vitro*. J Immunol 139:1902, 1987
37. Dunn CJ, Fleming WE: The role of interleukin-1 in the inflammatory response with particular reference to endothelial cell-leukocyte adhesion. In The Physiologic, Metabolic, and Immunologic Actions of Interleukin-1, edited by Kluger MJ, Oppenheim JJ, Powanda MC, p 45. New York, Alan R Liss, 1985
38. Elias RM, Johnston MG, Hayashi A, Nelson W: Decreased lymphatic pumping after intravenous endotoxin administration in sheep. Am J Physiol 253:183, 1987
39. Gahring L, Baltz M, Pepys MB, Daynes R: Effect of ultraviolet radiation on production of endothelial cell thymocyte-activating factor/interleukin 1 *in vivo* and *in vitro*. Proc Natl Acad Sci USA 81:1198, 1984
40. Galanos CO, Lüderitz O, Rietschel ET, Wesphal O: Newer aspects of the chemistry and biology of bacterial lipopolysaccharides, with special reference to their lipid A component. In Biochemistry of Lipids II, edited by Goodwin TW, p 239. Baltimore MD, University Park Press, 1977
41. Gamble RJ, Harlan JM, Klebanoff SJ, Lopez AF, Vadas MA: Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 82:8667, 1985
42. Gemmell CG: Comparative studies of the nature and biological activities of bacterial enterotoxins. J Med Microbiol 17:217, 1984
43. Goerd S, Zwadlo G, Schlegel R, Hagemeier H-H, Sorg C: Characterization and expression kinetics of an endothelial cell activation antigen present in vivo only in acute inflammatory tissues. Exp Cell Biol 55:117, 1987
44. Grantstein RD, Margolis R, Mizel SB, Sauder DN: *In vivo* inflammatory activity of epidermal cell-derived thymocyte-activating factor and recombinant interleukin 1 in the mouse. J Clin Invest 77:120, 1986
45. Habicht GS, Beck G: IL-1 is an endogenous mediator of acute inflammation and of the local Shwartzman reaction. In The Physiologic, Metabolic, and Immunologic Actions of Interleukin-1, edited by Kluger MJ, Oppenheim JJ, Powanda MC, p 13. New York, Alan R Liss, 1985
46. Hacker J, Hughes C, Hof H, Goebel W: Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. Infect Immunol 42:57, 1983
47. Hamilton SM, Johnston MG, Fong A, Pepevnak C, Semple JL, Movat HZ: Relationship between increased vascular permeability and extravascular albumin clearance in rabbit inflammatory responses induced with *Escherichia coli*. Lab Invest 55:580, 1986
48. Hanson DF, Murphy PA, Windle BE: Failure of rabbit neutrophils to secrete endogenous pyrogen when stimulated with staphylococci. J Exp Med 151:1360, 1980
49. Hay JB, Johnston MG, Hobbs BB, Movat HZ: The use of radioactive microspheres to quantitate hyperemia in dermal inflammatory sites. Proc Soc Exp Biol Med 150:641, 1975
50. Holmgren J: Toxins affecting intestinal transport processes. In The Virulence of *Escherichia coli*, edited by Sussman M, p 177. London, Academic Press, 1985
51. Issekutz AC, Bhimji S: Role of endotoxin in the leukocyte infiltration accompanying *Escherichia coli* inflammation. Infect Immun 36:558, 1982
52. Issekutz AC, Bhimji S, Bertolussi R: Effect of immune serum or polymyxin B on *Escherichia coli*-induced inflammation and vascular injury. Infect Immun 36:548, 1982
53. Issekutz AC, Megyeri P: Induction of leukocyte infiltration by endotoxin. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 24. Basel, S Karger AG, 1987
54. Issekutz AC, Megyeri P, Issekutz TB: Role of macrophage products in endotoxin-induced polymorphonuclear leukocyte accumulation during inflammation. Lab Invest 56:49, 1987
55. Issekutz AC, Movat HZ: Quantitation of neutrophil infiltration *in vivo*. Immunol Lett 1:27, 1979
56. Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of rabbit neutrophil leukocyte accumulation in the skin in response

to chemotactic agents and *Escherichia coli*. Lab Invest 42:310, 1980
57. Issekutz AC, Movat HZ: The effect of vasodilatory prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. Am J Pathol 107:300, 1982
58. Issekutz AC, Movat KW, Movat HZ: Enhancement of vascular permeability and haemorrhage-inducing activity of rabbit $C5a_{desArg}$: probable role of polymorphonuclear leukocyte lysosomes. Clin Exp Immunol 41:512, 1980
59. Issekutz AC, Ripley M, Rochon Y, Pi-Jimenez E, Wright B: A role for hemolysin in *Escherichia coli*-induced inflammation in granulocytopenic rabbits. J Infect Dis 150:925, 1984
60. Issekutz AC, Wankowicz Z: Synergy between tumor necrosis factor (TNFα) and interleukin 1 (IL-1) in the induction of polymorphonuclear leukocyte emigration (abstr). Fed Proc 46:737, 1987
61. Issekutz TB, Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of monocyte migration into acute inflammatory tissue. Am J Pathol 103:74, 1981
62. Jeynes BJ, Issekutz AC, Issekutz TB, Movat HZ: Quantitation of platelets in the microcirculation. Measurement of indium-111 in microthrombi induced in rabbits by inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 165:445, 1980
63. Johnston MG: Interaction of inflammatory mediators with the lymphatic vessel. Pathol Immunopathol Res 6:177, 1987
64. Johnston MG, Hay JB, Movat HZ: Kinetics of prostaglandin production in various inflammatory lesions, measured in draining lymph. Am J Pathol 95:225, 1979
65. Kampschmidt RF: Leukocytic endogenous mediator/endogenous pyrogen. In Infection: The Physiologic and Metabolic Responses of the Host, edited by Powanda MC, Canonico PG, p 403. Amsterdam, Elsevier Science Publisher, 1981
66. Klemm P: Fimbrial adhesions of *Escherichia coli*. Rev Infect Dis 7:321, 1985
67. Koj A: Biologic functions of acute-phase proteins. In The Acute-Phase Response to Injury and Infection, edited by Gordon AH, Koj A, p 145. Amsterdam, Elsevier, 1985
68. Kopaniak MM, Issekutz AC, Burrowes CE, Movat HZ: The quantitation of hemorrhage in the skin: measurement of hemorrhage in the microcirculation in inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 163:126, 1980
69. Kopaniak MM, Issekutz AC, Movat HZ: Kinetics of acute inflammation induced by *E. coli* in rabbits. Quantitation of blood flow, enhanced vascular permeability, hemorrhage and leukocyte accumulation. Am J Pathol 98:485, 1980
70. Kopaniak MM, Movat HZ: Kinetics of acute inflammation induced by *Escherichia coli* in rabbits. II. The effect of hyperimmunization, complement depletion, and depletion of leukocytes. Am J Pathol 110:13, 1983
71. Libby P, Ordovas JM, Auger KR, Robbin HH, Biriny LK, Dinarello CA: Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult vascular endothelial cells. Am J Pathol 124:179, 1986
72. Libby P, Ordovas JM, Birinyi LK, Auger KR, Dinarello CA: Inducible interleukin 1 expression in human vascular smooth muscle cells. J Clin Invest 78:1432, 1986
73. Luscinskas FW, Bevilacqua MP, Brock AF, Arnaout MA, Gimbrone MA Jr: Endothelial-leukocyte adhesion: Contributions of endothelial dependent and leukocyte-dependent mechanisms (abstr). Fed Proc, in press 1988
74. Marasco WA, Phan SH, Krutsch H, Showell HJ, Feltner DE, Nairn R, Becker EL, Ward PA: Purification and identification of formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J Biol Chem 259:5430, 1984
75. McCabe WR: Gram negative bacteremia. In Infectious Diseases and Medical Microbiology, edited by Braude AI, Davis CE, Fierer J, p 1177. Philadelphia, WB Saunders, 1986
76. McComb DJ, Cybulsky MI, Movat HZ: PMN emigration: protein synthesis dependent and independent mechanisms (abstr). Fed Proc 46:1390, 1987
77. McGowan JE, Barnes MW, Finland M: Bacteremia at Boston City Hospital: occurrence and mortality during 12 selected years (1935–72), with special reference to hospital-acquired cases. J Infect Dis 132:316, 1975
78. Mooi FR, De Graaf FK: Molecular biology of fimbriae of enterotoxigenic *Escherichia coli*. Curr Topics Microbiol Immunol 118:119, 1985
79. Morrison DC, Ulevitch RJ: The effects of bacterial endotoxins on host mediation systems. Am J Pathol 93:526, 1978
80. Movat HZ: Microcirculation in disseminated intravascular coagulation induced by endotoxin. In Handbook of Physiology, Vol IV, The Cardiovascular System, edited by Renkin EN, Michel CC, p 1047. Bethesda MD, American Physiological Society, 1984
81. Movat HZ: The Inflammatory Reaction, Amsterdam, Elsevier, 1985
82. Movat HZ, Burrowes CE: Elicitation of the Shwartzman reaction by a combination of endotoxin and agents which activate the complement system: microvascular events. In Immunopharmacology of Endotoxicosis, edited by Agarwal MK, Yoshida M, p 197. Berlin/New York, Walter de Gruyter and Co, 1984
83. Movat HZ, Burrowes CE: Local Shwartzman reaction: endotoxin-mediated inflammatory and thrombo-hemorrhagic lesions. In Handbook of Endotoxin, Volume 3, Cellular Biology of Endotoxin, edited by Berry LJ, p 260. Amsterdam, Elsevier, 1985
84. Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA: Role of complement, interleukin-1 and tumor necrosis factor in a local Shwartzman-like reaction. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 69. Basel, S Karger AG, 1987
85. Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA: Acute inflammation and a Shwartzman-like reaction induced by interleukin 1 and tumor necrosis factor. Synergistic action of the cytokines in the induction of inflammatory and microvascular injury. Am J Pathol 129:463, 1987
86. Movat HZ, Cybulsky MI, Colditz IG, Chan MKW, Dinarello CA: Acute inflammation in gram negative infection. Role of endotoxin, interleukin 1, tumor necrosis factor and neutrophils. Fed Proc 46:97, 1987
87. Movat HZ, Jeynes BJ, Wasi S, Movat KW, Kopaniak MM: Quantitation of the development and progression of the local Shwartzman reaction. In Bacterial Endotoxins and Host Response, edited by Agarwal MK, p 179. Amsterdam, Elsevier, 1980
88. Movat HZ, Rettl C, Burrowes CE, Johnston MG: The *in vivo* effect of leukotriene B_4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement ($C5a_{desArg}$) and enhancement by prostaglandin E_2. Am J Pathol 115:233, 1984
89. Movat HZ, Wasi S: Severe microvascular injury induced by lysosomal releasates of human polymorphonuclear leukocytes. Increase in vasopermeability, hemorrhage, and microthrombosis due to degradation of subendothelial and perivascular matrices. Am J Pathol 121:404, 1985
90. Müller-Berghaus G, Lasch H-G: Microcirculatory disturbances induced by generalized intravascular coagulation. In Handbook of Experimental Pharmacology, Vol 16, Experimental Production of Diseases: Heart and Circulation, edited by Schmier J, Eichler O, p 429. Berlin, Springer-Verlag, 1975
91. Nachman RL, Hajjar KA, Silverstein RL, Dinarello CA: Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. J Exp Med 163:1260, 1986
92. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D: Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363, 1986
93. Nawroth PP, Stern DM: Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 163:740, 1986
94. Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank MM, Burke JF, Gelfand JA: C5a induction of interleukin 1. Synergistic effect with endotoxin or interferon-γ. J Immunol 139:2635, 1987
95. Okusawa S. Gelfand JA, Ikejima T, Connolly RJ, Dinarello CA: Interleukin-1 induces a shock-like state in rabbits: synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J Clin Invest, in press 1988
96. Parry SH, Rooke DM: Adhesions and colonization factors of *Escherichia coli*. In The Virulence of *Escherichia coli*, edited by Sussman M, p 79. London, Academic Press, 1985
96a. Pettipher ER, Higgs GA, Henderson B: Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in

the synovial joint. Proc Natl Acad Sci USA 83:8749, 1986
97. Pober JS, Bevilacqua MP, Mendrick DL, Lapierre LA, Fiers W, Gimbrone MA Jr: Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J Immunol 136:1680, 1986
98. Pober JS, Lapierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA Jr: Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. J Immunol 138:3319, 1987
99. Pohlman TH, Stanness KA, Beatty PG, Ochs HD, Harlan JM: An endothelial cell surface factor (s) induced *in vitro* by lipopolysaccharide, interleukin 1 and tumor necrosis factor-increases neutrophil adherence by a CDw18-dependent mechanisms. J Immunol 136:4548, 1986
100. Rogers DE: The changing pattern of life-threatening microbial disease. N Engl J Med 261:677, 1959
101. Ruff MR, Gifford GE: Tumor necrosis factor. Lymphokines 2:235, 1981
102. Sanders CC, Sanders WE Jr: Microbial resistance to newer generation β-lactam antibiotics: clinical and laboratory implications. J Infect Dis 151:399, 1981
103. Schleimer RP, Rutledge BK: Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin and tumor-promoting phorbol diesters. J Immunol 136:649, 1986
104. Shwartzman G: Phenomenon of Local Tissue Reactivity and its Immunological, Pathological and Clinical Significance, New York, Paul Hoeber, 1937
105. Sinanan M, Maier RV, Corrico J: Labarotomy for intra-abdominal sepsis in an intensive care unit. Arch Surg 119:652, 1985
106. Springer TA, Dustin ML, Kishimoto TK, Marlin SD: Lymphocyte function-associated LFA-1, CD2, and LFA-3 receptors of the immune system. Annu Rev Immunol 5:223, 1987
107. Stern DM, Bank I, Nawroth PP, Cassimeris J, Kisiel W, Fenton JW II, Dinarello C, Chess L, Jaffe EA: Self-regulation of procoagulant events on the endothelial cell surface. J Exp Med 162:1223, 1985
108. Stern D, Nawroth P, Handley D, Kisiel W: An endothelial cell-dependent pathway of coagulation. Proc Natl Acad Sci USA 82:2523, 1985
109. Stetson CA Jr: Similarities in the mechanisms determining the Arthus and Shwartzman phenomenon. J Exp Med 94:347, 1951
110. Stetson CA Jr, Good RA: Studies on the mechanisms of the Shwartzman phenomenon: Evidence for the participation of polymorphonuclear leukocytes in the phenomenon. J Exp Med 93:49, 1951
111. Tiku K, Tiku ML, Skosey JL: Interleukin 1 production by human polymorphonuclear leukocytes. J Immunol 136:3677, 1986
112. Timmis KN, Boulnois GJ, Bitter-Sauermann D, Cabello FC: Surface components of *Escherichia coli* that mediate resistance to the bactericidal activities of serum and phagocytes. Curr Top Microbiol Immunol 118:197, 1985
113. Tobin MK, Grenvik A: Nosocomial lung infection and its diagnosis. Crit Care Med 12:191, 1984
114. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark JW, Hariri RJ, Fahey III TJ, Zentella A, Albert JD, Shires GT, Cerami A: Shock and tissue injury induced by recombinant human cachectin. Science 234:470, 1986
115. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Exp Biol Med 133:1384, 1970
116. Uhlin BE, Baga M, Goranson M, Lindberg FP, Lund B, Normark S: Genes determining adhesin formation in uropathogenic *Escherichia coli*. Curr Top Microbiol Immunol 118:163, 1985
117. Ulich TR, del Castillo J, Keys M, Granger GA, Ni R-X: Kinetics and mechanisms of recombinant human interleukin 1 and tumor necrosis factor-α-induced changes in circulating numbers of neutrophils and lymphocytes. J Immunol 139:3406, 1987
118. Warner SJC, Auger KR, Libby P: Interleukin 1 induces interleukin 1. II. Recombinant human interleukin 1 induces interleukin 1 production by adult human vascular endothelial cells. J Immunol 139:1911, 1987
119. Welch RA, Falkow S: Characterization of *Escherichia coli* hemolysins conferring quantitative differences in virulence. Infect Immun 43:156, 1984
120. Westphal O, Jann K, Himmelspach K: Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. Prog Allergy 33:9, 1983
121. Windle BE, Murphy PA, Cooperman S: Rabbit polymorphonuclear leukocytes do not secrete endogenous pyrogen or interleukin 1 when stimulated by endotoxin, polyinosine; polycytosine, or muramyl dipeptide. Infect Immunol 39:1142, 1983
122. Yoshinaga M, Goto F, Goto K, Ohkawara S, Kitamura M, Mori S: Triggering of polymorphonuclear leukocytes to produce interleukin-1 at the inflammatory site. In Leukocyte Emigration and its Sequelae, edited by Movat HZ, p 169. Basel, S Karger AG, 1987

STIC-ILL

QP1. W533
Adams

From: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

JOURNAL OF CELLULAR PHYSIOLOGY 163:19–29 (1995)

Reciprocal Induction of Tumor Necrosis Factor-α and Interleukin-1β Activity Mediates Fibronectin Synthesis in Coronary Artery Smooth Muscle Cells

SILVANA MOLOSSI, NADINE CLAUSELL, AND MARLENE RABINOVITCH*
Division of Cardiovascular Research, Research Institute, The Hospital For Sick Children, Toronto, Ontario, Canada, M5G 1X8, and Departments of Pediatrics, Pathology and Medicine, University of Toronto, Toronto, Ontario, Canada

We previously demonstrated an immune-inflammatory response associated with increased expression of interleukin (IL)-1β and fibronectin in graft coronary arteriopathy in piglets following heterotopic heart transplant. Further studies showed that increased endogenously produced IL-1β was upregulating fibronectin production by donor coronary artery (CA) smooth muscle cells (SMC). Since coinduction of IL-1β and tumor necrosis factor (TNF)-α has been shown in other systems, we investigated the possible interaction between these cytokines in regulating fibronectin production in CA SMC. First, we documented increased TNF-α expression in vivo in donor compared to host CA. Next, synthesis of fibronectin was measured in host and donor CA SMC following [^{35}S]-methionine radiolabeling and gelatin-sepharose extraction. As previously shown with IL-1β, increased donor CA SMC fibronectin synthesis was reduced to host levels in the presence of TNF-α antibodies, and exogenous TNF-α upregulated fibronectin synthesis in host CA SMC to levels in donor cells. In normal CA SMC, TNF-α-stimulated fibronectin production was downregulated to or below control levels in the presence of IL-1β antibodies. Likewise, IL-1β-stimulated fibronectin synthesis was downregulated to control levels when TNF-α neutralizing antibodies were added. Combining TNF-α and IL-1β enhanced fibronectin production over that observed with either cytokine alone, but was not additive. Thus, our studies suggest that vascular SMC fibronectin synthesis is regulated by reciprocal induction of IL-1β and TNF-α activity and provide the first demonstration of a 'cytokine loop' modulating matrix production.

The development of an accelerated form of coronary artery intimal thickening has become a major complication in patients following cardiac transplantation (Gao et al., 1990; Uretsky et al., 1987). This arteriopathy is associated with an immune-inflammatory response in the vascular wall, suggesting a mechanism for neointimal formation whereby recruitment of immune-reactive cells, and their subsequent release and induction of various cytokines and growth factors, results in smooth muscle migration and proliferation in the subendothelium and accumulation of extracellular matrix (Libby et al., 1989; Salomon et al., 1991). Previous studies in our laboratory have, in fact, shown that the development of graft arteriopathy in piglets following heterotopic heart transplantation is associated with the adhesion and transendothelial migration of T cells, subendothelial accumulation of fibronectin, and increased expression of interleukin (IL)-1β in the allograft coronary arteries (Clausell et al., 1993). In cultured allograft (donor) coronary artery endothelial and smooth muscle cells, we further established that the increased synthesis of fibronectin was modulated by increased endogenously produced IL-1β (Clausell and Rabinovitch, 1993; Molossi et al., 1993).

Gene expression of a variety of cytokines has been demonstrated in rejecting allografts, and these cytokines include IL-1β (Fanslow et al., 1991) and tumor necrosis factor (TNF)-α (Arbustini et al., 1991; Imagawa et al., 1991). In immunohistochemical studies using specimens from human cardiac biopsies, TNF-α was shown to be present and appeared to be related to the degree of rejection (Arbustini et al., 1991). Tumor necrosis factor-α is a modulator of extracellular matrix synthesis (Kaji et al., 1993; Mauviel et al., 1988; Varani et al., 1989) and, recently, our group has described this function in the setting of allograft arteriopathy. Using a rabbit heterotopic cardiac transplant model, we have

Received March 28, 1994; accepted August 30, 1994.

*To whom reprint requests/correspondence should be addressed at Division of Cardiovascular Research, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

shown that neutralizing TNF-α activity by using the TNF-α soluble receptor reduced the incidence and severity of lesions related to the allograft arteriopathy, and this was associated with decreased expression of fibronectin. Furthermore, there was downregulation of the immune-inflammatory response in the allograft coronary arteries (Clausell et al., 1994).

In the setting of intra-graft reactions, cytokines can be expected to function as mediators of cell to cell communication in the immune-inflammatory reaction (Hancock et al., 1991). Indeed, these peptides can interact in various manners and induce production of other cytokines, a process which has been referred to as a "cytokine cascade" by several authors (Ford et al., 1990), and which could potentially initiate a chain of events on the surface of the vessel wall (Nawroth et al., 1986). Specifically, TNF-α induces IL-1 synthesis (Dinarello et al., 1986; Le and Vilcek, 1987; Nawroth et al., 1986) by stabilizing IL-1 mRNA, a process which requires protein kinase C activity (Gorospe et al., 1993). It has been further shown that blockade of the 55-kDa TNF receptor inhibits the TNF-mediated activation of the IL-1 transcription factor NF-κB (Kruppa et al., 1992). Likewise, IL-1 is known to induce TNF-α synthesis (Bethea et al., 1992), and also its own production (Dinarello, 1991; Warner et al., 1987). Both IL-1α and TNF-α have also been shown to exert synergistic effects in mediating cellular functions, i.e., enhancing production of myeloid colony-stimulating factors by cultured human bone marrow and cloned stromal cells (Caldwell and Emerson, 1994). Moreover, TNF-α has been shown to enhance fibronectin synthesis in normal melanocytes and malignant melanoma cells when in combination with gamma interferon (Varani et al., 1989). An interrelation between the expression of these two cytokines was suggested by our studies in the experimental allograft arteriopathy in that TNF-α blockade with TNF-soluble receptor downregulates both IL-1β as well as its own expression (Clausell et al., 1994).

Based on the above information, we hypothesized that increased TNF-α is associated with the development of graft arteriopathy by inducing increased fibronectin synthesis via upregulation of IL-1β. Our present studies confirmed that neutralization of TNF-α activity in vitro led to downregulation of donor coronary artery smooth muscle cell fibronectin synthesis, whereas stimulation of host (native) cells with exogenous TNF-α induced progressive increase in fibronectin in a dose-response manner. We further demonstrated that TNF-α and IL-1β are largely interdependent, but there may also be some separate effects on their stimulation of normal coronary artery smooth muscle cell fibronectin production given the enhanced synthesis of this protein with combined cytokine stimulation. These novel in vitro findings suggest that a reciprocal induction of the activity of these cytokines regulates fibronectin production in inflammatory states, specifically related to smooth muscle cell migration and neointimal formation in blood vessels.

MATERIALS AND METHODS

Experimental animal model

The experimental protocol used was approved by the Animal Care Committee of The Hospital for Sick Children, Toronto. The animal model has been previously described in detail (Clausell et al., 1993; Clausell and Rabinovitch, 1993). Briefly, it consisted of a heterotopic cardiac transplant performed in outbred piglets between 8 and 12 weeks of age and approximately 20 kg in weight. Both host and donor piglets were purchased from different farms, favouring human leukocyte antigen (HLA)-mismatch. The animals received penicillin-G (150,000 units IM) and standard post-operative care in compliance with the Principles of Laboratory Animal Care formulated by the Canadian National Society of Medical Research. The piglets received immunosuppressive therapy with sub-therapeutic dose of Cyclosporine A (CsA) (Sandimune, Sandoz, East Hanover, NJ) (10 mg/kg/day for 5 days). All piglets had electrocardiographic monitoring performed daily by telemetric assessment (Pacesetter Electrocardiogram, Syalam, CA) to identify signs of progressive rejection as previously described (Koike et al., 1988). Experimental animals were studied at 10 days after transplantation since there was light and electron microscopic evidence consistent with the post-cardiac transplant coronary arteriopathy as previously described (Clausell et al., 1993). Myocardial rejection grades II and III were observed in the transplanted hearts at the time of the sacrifice (Clausell et al., 1993).

For experiments in which normal porcine coronary artery tissue from non-transplanted piglets was used, hearts were obtained from animals used in experimental procedures not involving the cardiopulmonary system in the Animal Facility Laboratory at the Hospital For Sick Children, Toronto. These piglets were matched in age and weight to those used in the transplant procedures.

Harvest of coronary artery tissue and propagation of smooth muscle cells

Coronary artery segments, approximately 1 cm from the origin at the aorta and 4 cm in length, were removed either from both the host and the donor hearts following heterotopic transplant or from normal non-transplant piglet hearts and rinsed in D-PBS (Gibco, Burlington, ON) with 3% antibiotics/antimycotics (Gibco). The most distal 1 cm of these segments was fixed for light and electron microscopy (0.5 cm, respectively) to analyze structural changes previously described (Clausell et al., 1993) and to perform immunohistochemical studies. The proximal 3 cm of the coronary arteries were used for harvest and culture of cells.

After removing the endothelium, explants from the media were used to propagate the smooth muscle cells (Ross, 1971) used in the present studies. The cells were cultivated in medium 199 with 25 mM HEPES buffer and glutamine (Gibco) and 1% antibiotics/antimycotics (Gibco) and 10% fetal bovine serum (FBS) (Intergen, New York, NY). Cells were passaged 1:2 non-enzymatically into 25 ml flasks and all experiments were carried out using cells at passages 2 and 3. Only cells with the "hills and valleys" phenotype on phase contrast light microscopy (Nikon Diaphot, Nikon, Mississauga, ON) were considered smooth muscle cells and were further positively identified by immunofluorescence using monoclonal antibodies specific to smooth muscle actin (Enzo, New York, NY) (Gown et al., 1986).

Immunohistochemical studies of TNF-α in host and donor coronary arteries

Immunoperoxidase staining to determine TNF-α expression in host and donor coronary arteries was performed using fresh frozen sections. The specimens were dried at room temperature for 1 hr and fixed for 20 min in acetone, followed by immersion in 1% hydrogen peroxide solution in methanol to block endogenous peroxidase activity. After a non-specific blocking step with 10% normal goat serum (Sigma, St. Louis, MO), the sections were incubated overnight at 4°C with a monoclonal anti-rabbit TNF-α antibody (IgG_1subtype in ascites fluid, a kind gift from Dr. Hideo Nariuchi, University of Tokyo, Japan) in a 1:100 dilution. The sections were then rinsed and incubated for 45 min at room temperature with a 1:50 dilution of a goat anti-mouse peroxidase conjugated antibody (Bio-Rad, Richmond, CA), washed in PBS, and developed with diaminobenzidine (DAB) (Sigma).

Synthesis of fibronectin

To determine synthesis of fibronectin, confluent smooth muscle cells had culture medium replaced with 2 ml serum-free medium (SFM) to which 20 μCi/ml of [^{35}S]-methionine (Amersham, Oakville, ON) was added for 24 hr. After this period, the culture medium was removed and saved in the presence of protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF) (Kodak, Rochester, NY), 10 mM N-ethylmaleamide (Sigma), and 10 mM EDTA (Sigma). The cell layers were removed and saved in D-PBS (Gibco) and stored at 70°C. To extract fibronectin, 400 μl aliquots of medium and cells were applied to a gelatin 4B-Sepharose (Pharmacia, Uppsala, Sweden) micro-column (Wrana et al., 1988). After washing the micro-column with a high-salt buffer, the fibronectin retained was eluted by boiling the samples for 5 min in 200 μl sodium dodecyl sulfate (SDS)-sample buffer and resolved on 5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The gels were prepared for autoradiography and for quantitative analysis by treatment with En^{3}Hance (DuPont, Boston, MA) for 1 hr, rinsed in water for 30 min, dried on a Bio-Rad Model 443 slab dryer (Bio-Rad), and exposed to Kodak X-OMAT AT-5 film for 4 days at −70°C. Using the autoradiograph as a template, the doublet 220-kDa band corresponding to fibronectin was cut and radioactivity determined by liquid scintillation spectrometry. Fibronectin isolated by affinity chromatography on gelatin-Sepharose constituted the majority (>95%) present in the aliquot of medium passed through the micro-column, as judged by scintillation counting and the absence of an autoradiographically detectable band at 220 kDa molecular weight when the column flow-through and washes were collected and run on 5% SDS-PAGE under reducing conditions. Positive determination of this band as fibronectin was previously confirmed in our laboratory by western immunoblot (Boudreau and Rabinovitch, 1991) and negative blots were seen on SDS-PAGE following re-constitution of the flow-through and washes. Furthermore, a minor band migrating at 70 kDa molecular w ight was observed, which represented gelatinase and demonstrated the effectiveness of this affinity chromatography procedure as described previously (Wrana et al., 1988). In all experiments, due to variability of cellular response and radioactivity half-life, the effect of either stimulation or inhibition of fibronectin synthesis was compared to baseline levels.

Experiments using exogenous IL-1β and TNF-α

At confluence, normal coronary artery smooth muscle cells had the culture medium replaced with fresh serum-free medium (2 ml) and were labeled with [^{35}S]-methionine (20 μCi/ml) (Amersham). This was followed by a 24 hr incubation period at 37°C in the presence of either increasing doses of recombinant human IL-1β (Upjohn, Kalamazoo, MI), ranging from 0.5 to 100 ng/ml, or recombinant human TNF-α (R&D Systems, Minneapolis, MN), in doses ranging from 0.05 to 10 ng/ml. Control samples were also treated with normal rabbit IgG at doses of 10 and 100 ng/ml, since further experiments using neutralizing antibodies require a normal immunoglobulin as the appropriate control (Mathison et al., 1988; Piguet et al., 1992).

Experiments neutralizing TNF-α activity

Smooth muscle cells at confluence were washed and incubated in serum free medium (2ml), and the cells were labeled with [^{35}S]-methionine (20 μCi/ml) (Amersham) and incubated at 37°C for 24 hr in the presence of neutralizing TNF-α antibodies (monoclonal mouse anti-human IgG) (R&D Systems). In the experiments using host and donor coronary artery smooth muscle cells from the animals submitted to cardiac transplantation, TNF-α antibodies (goat anti-human IgG) (R&D Systems) were used in increasing doses ranging from 0.05 to 1 μg/ml and the control had normal rabbit IgG (Dako) added at a concentration of 10 ng/ml. In the experiments using normal coronary artery smooth muscle cells, TNF-α antibodies (R&D Systems) were used in doses of 0.05 and 0.5 μg/ml since these dosages were shown to decrease fibronectin production in the presence of either control rabbit IgG (10 ng/ml) (Dako, Glostrup, Denmark) or exogenous TNF-α (R&D Systems) in doses of 5 and 10 ng/ml. These same doses of TNF-α antibodies (R&D Systems) were also used in the presence of exogenous IL-1β (Upjohn) at a dose of 100 ng/ml. The concentration range of exogenous TNF-α (R&D Systems) and IL-1β (Upjohn) was chosen after pilot studies showed an effect in upregulating fibronectin synthesis from control (IgG) levels.

Experiments neutralizing IL-1β activity

To neutralize porcine IL-1β activity in normal coronary artery smooth muscle cells, rabbit anti-porcine IL-1β antisera (a kind gift from Dr. J. Saklatvala, AFRC Babraham Institute, Cambridge, England) was used at a dilution of 1:50 (Saklatvala et al., 1985; Clausell and Rabinovitch, 1993), in the presence of either control rabbit IgG (Dako) (10 ng/ml) or exogenous IL-1β or TNF-α (R&D Systems). Interleukin-1β (Upjohn) was added at a dose of 100 ng/ml and TNF-α (R&D Systems) at the doses of 5 and 10 ng/ml. The doses were chosen according to our previous studies in which neutralization of cytokine activity or stimulation of fibronectin in vascular cells was shown.

Experiments assessing synergism between IL-1β and TNF-α

At confluence, normal coronary artery smooth muscle cells had the culture medium replaced with fresh

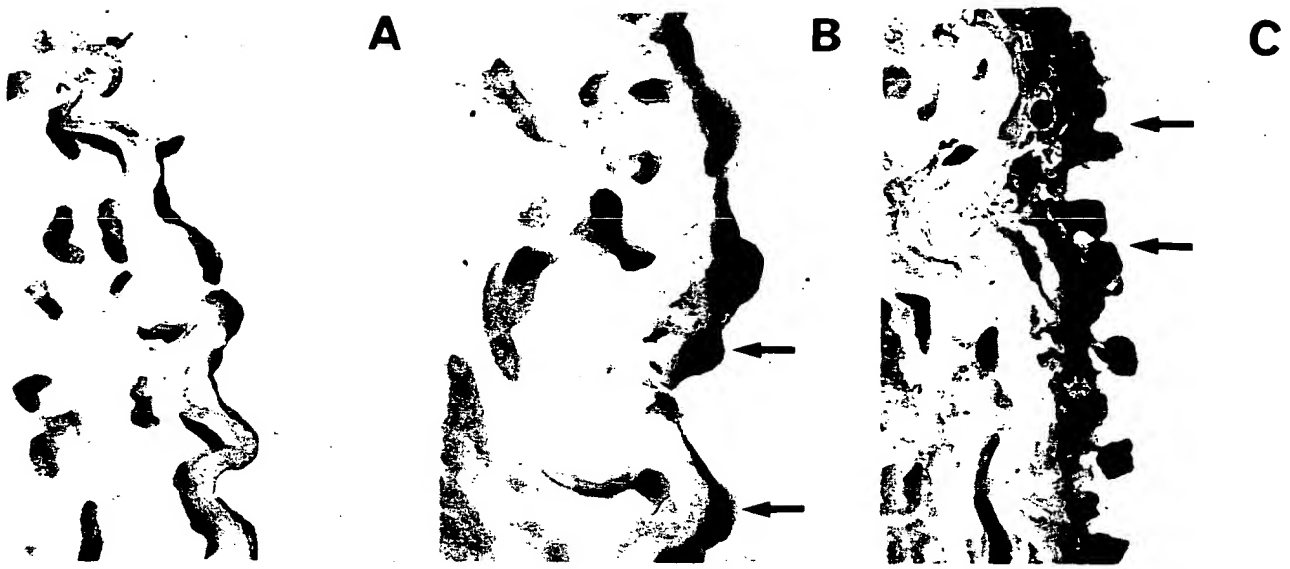


Fig. 1. Representative photomicrographs of immunoperoxidase staining for TNF-α in host (A) and donor (B and C) porcine coronary arteries from a piglet 10 days following cardiac transplantation. A basal level of expression of TNF-α is observed in the host coronary artery along the endothelial surface (A), whereas stronger immunostaining is appreciated in donor vessels on the endothelium (B and C) and also in the medial layer of some allograft coronary arteries (C). The increased expression of TNF-α may be due to endogenous production by both endothelial and smooth muscle cells, and also by immune-inflammatory cells seen attached to the endothelial surface (C). Original magnification 100×.

serum-free medium (2 ml) and were labeled with [^{35}S]-methionine (20 μCi/ml) (Amersham). This was followed by a 24 hr incubation period at 37°C in the presence of either recombinant human IL-1β (Upjohn), at a dose of 100 ng/ml, or recombinant human TNF-α (R&D Systems), at doses of 5 and 10 ng/ml, or both cytokines together at the specified doses. Control samples were either non-treated or treated with normal rabbit IgG (Dako) at doses of 10 and 100 ng/ml. As well, we carried out experiments in which we added normal rabbit IgG to samples treated with cytokines either alone or in combination, in order to rule out a possible effect of IgG on the net production of fibronectin in the presence of exogenous stimulation.

DNA standardization

All values related to fibronectin synthesis were standardized by determining DNA content as previously described (Le Pecq, 1971) and results are expressed as cpm/100 ng DNA. Briefly, duplicates of 100 μl aliquot cell suspension were removed and added to a mixture of 300 μl of Tris buffer (100 mM NaCl (Sigma), 10 mM Trizma pH 8.0 (Sigma), adjusted to pH 7.0), and 140 μl of 40% TCA. Following an incubation at 4°C for 15 min, the samples were centrifuged at 3,250 rpm for 10 min at 4°C. The supernatant was removed and 1.5 ml of 10 × TNE buffer [100 mM Tris base (Sigma), 10 mM EDTA (Sigma), 1 M NaCl (BDH, Toronto, ON), adjusted to pH 7.4]. Measurement of DNA binding to the fluorescence dye ethidium bromide (0.1% in 1 × TNE buffer) (Sigma) was d termined by spectrophotometry (Hitachi, F-400 Fluor scent Spectrophotometer). Values were standardized using calf thymus DNA (Sigma).

Statistical analysis

Data are expressed as mean ± SE in the Results and in the figure legends. The number of different experiments (cell harvests) used in each analysis is also indicated. In analyses related to values for fibronectin synthesis in normal coronary artery smooth muscle cells, one way analysis of variance (ANOVA) with post-hoc paired sub-group testing by Duncan's multiple range analysis was performed, taking into account P value correction for multiple comparisons. The differences were considered statistically significant at $P < 0.05$. In the first experiment comparing host and/or donor smooth muscle cells, the data represent the mean value of two different animals.

RESULTS

Localization of TNF-α in allograft coronary arteries

Since we had previously demonstrated increased expression of IL-1β in piglet donor coronary arteries and of both IL-1β and TNF-α in rabbit donor coronary arteries, we carried out immunohistochemical studies to confirm that TNF-α expression was also a feature of the arteriopathy in piglets. In vivo expression of TNF-α was examined in host and donor coronary arteries from nine different piglets following cardiac transplantation. Strong positive immunoperoxidase staining was evident in all donor coronary arteries compared to host vessels, where only faint staining along the endothelial surface could be seen (Fig. 1A). In all donor coronary arteries, intense immunostainig was appreciated in association with endothelial cells (arrows) (Fig. 1B), and also in areas where inflammatory cells (arrows) were

seen attached to the endothelium (Fig. 1C). Positive staining was also seen in the inner medial layers of some donor vessels (Fig. 1C). These coronary arteries had been previously characterized as showing positive immunostaining for inflammatory cells, IL-1β, and fibronectin in these locations (Clausell et al., 1993).

Regulation of fibronectin synthesis in allograft coronary artery smooth muscle cells by TNF-α

Having demonstrated increased expression of TNF-α in donor piglet coronary arteries and having shown that blocking this cytokine in a rabbit allograft model decreased expression of fibronectin, we carried out further in vitro studies to determine whether endogenous TNF-α might be mediating enhanced fibronectin production. Host and donor coronary artery smooth muscle cells from two different piglets (from which tissue was available for culture of smooth muscle cells) were exposed to increasing doses, from 0.05 to 1 μg/ml, of neutralizing TNF-α antibodies and fibronectin synthesis was assessed. Fibronectin levels in donor coronary artery smooth muscle cells were approximately 3.5-fold higher than levels observed in host cells, in keeping with our previous studies (Clausell and Rabinovitch, 1993). In this experiment, we observed a decrease in donor smooth muscle cell fibronectin synthesis with increasing doses of TNF-α neutralizing antibodies (Fig. 2A). Values were similar to those of the host cells, where fibronectin synthesis did not appear to be influenced by neutralizing TNF-α activity.

We next addressed the effect of exogenous TNF-α in upregulating fibronectin synthesis in host coronary artery smooth muscle cells. We incubated host cells with increasing doses of recombinant human TNF-α, ranging from 0.05 to 5 ng/ml, and assessed fibronectin synthesis. We observed, at concentrations of 1 and 5 ng/ml TNF-α, an increase in fibronectin synthesis in the cells harvested from both piglets studied and the graph (Fig. 2B) depicts mean values. Exogenous TNF-α did not further increase fibronectin synthesis in donor cells (data not shown), suggesting perhaps that maximum fibronectin production had been already achieved in these vascular cells.

Upregulation of fibronectin synthesis by TNF-α and IL-1β in normal coronary artery smooth muscle cells

Since TNF-α can induce IL-1β and IL-1β can induce TNF-α, and inasmuch as both cytokines were expressed in increased concentration and shown to modulate fibronectin synthesis in the allograft coronary arteries (Clausell et al., 1993, 1994), we investigated their potential reciprocal interaction in mediating fibronectin synthesis in vascular smooth muscle cells. In these experiments, for reasons of availability, we used normal coronary artery smooth muscle cells. We confirmed in nine different experiments, the effect of increasing doses of exogenous TNF-α, ranging from 0.05 to 10 ng/ml, on the production of fibronectin by normal coronary artery smooth muscle cells in vitro. A statistically significant 50–70% increase in fibronectin synthesis was observed with TNF-α at high doses (5 and 10 ng/ml) ($P < 0.05$) compared to control (IgG) levels (Fig. 3). Although fibronectin synthesis was not increased to the level observed in host cells in the presence of even a smaller dose of TNF-α (5 ng/ml) (Fig. 2B), we did see consistent upregulation of fibronectin production in normal coronary artery smooth muscle cells at 5 and 10 ng/ml doses of TNF-α.

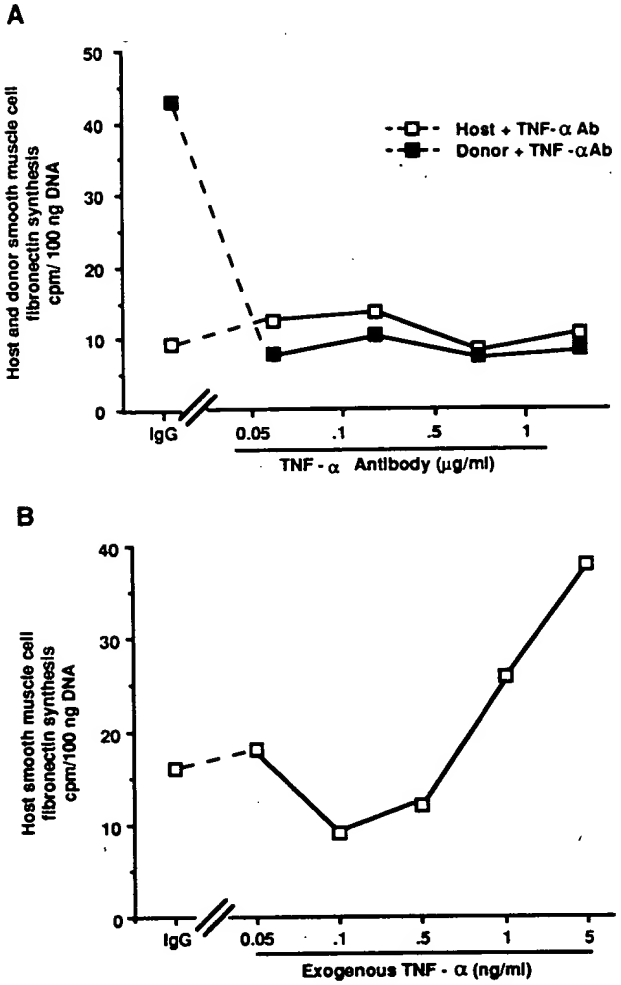


Fig. 2. Fibronectin synthesis in coronary artery smooth muscle cells from transplanted piglets. (A) Effect of neutralization with TNF-α antibodies on steady state levels of fibronectin. After labeling coronary artery smooth muscle cells with [^{35}S]-methionine for 24 hr, in the presence of increasing doses of neutralizing TNF-α antibody a progressive decrease in donor coronary artery smooth muscle cell fibronectin synthesis to the level of the host is observed in a dose-response manner. The host cell fibronectin synthesis did not appear to be influenced by the neutralizing antibody. Values depicted in the graph represent the mean value for fibronectin synthesis in cells from two different experimental animals. (B) Representative dose-response curve of host coronary artery smooth muscle cell fibronectin synthesis to increasing doses of human recombinant TNF-α. After labeling the cells with [^{35}S]-methionine for 24 hr in the presence of increasing doses of exogenous TNF-α, a progressive increase at the doses of 1 and 5 ng/ml in host coronary artery smooth muscle cell fibronectin synthesis recovered in the cell culture medium was observed. Values depicted in the graph represent the mean value for fibronectin synthesis in cells from two different experimental animals.

Likewise, in six different experiments, recombinant human IL-1β was added to normal coronary artery smooth muscle cells in vitro at doses ranging from 0.5 to 100 ng/ml, and fibronectin synthesis was assessed.

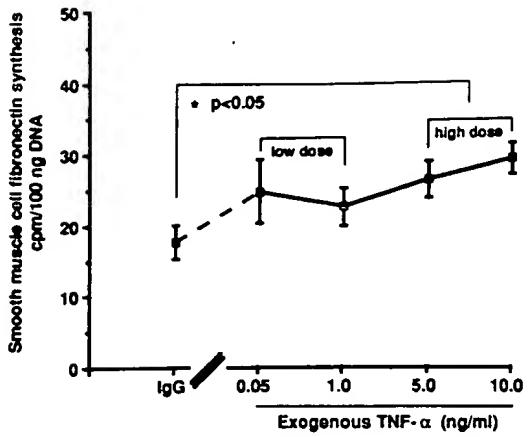


Fig. 3. Dose-response curve of normal coronary artery smooth muscle cell fibronectin synthesis to increasing doses of exogenous TNF-α. After labeling the cells with [^{35}S]-methionine for 24 hr in the presence of increasing doses of exogenous TNF-α, an increase in fibronectin synthesis recovered in the cell medium, relative to the control IgG-treated sample, was observed. Fibronectin levels were enhanced by 50 to 70% at high doses, i.e., 5 and 10 ng/ml ($P < 0.05$). Values depicted in the graph represent mean values ± SE from nine different experiments.

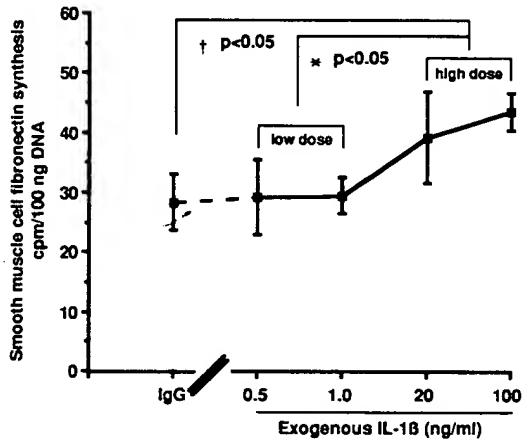


Fig. 4. Dose-response curve of normal coronary artery smooth muscle cell fibronectin synthesis to increasing doses of exogenous IL-1β. Following cell labeling with [^{35}S]-methionine for 24 hr in the presence of increasing doses of exogenous IL-1β, an increase in fibronectin synthesis recovered in the cell medium, relative to the control IgG-treated sample, was observed. Fibronectin levels were significantly enhanced by 50% at high concentrations of IL-1β, i.e., 20 and 100 ng/ml ($P < 0.05$). Values depicted in the graph represent mean values ± SE from six different experiments.

Fibronectin production in this system was significantly increased by 50% from control (IgG) values and low doses (0.5 and 1.0 ng/ml) of IL-1β compared to high doses (20 and 100 ng/ml) of IL-1β ($P < 0.05$) (Fig. 4).

Effect of TNF-α and IL-1β neutralizing antibodies on normal coronary artery smooth muscle cell fibronectin synthesis

To confirm the specific effects of both TNF-α and IL-1β in increasing fibronectin synthesis, we designed experiments blocking their respective activities and assessed whether fibronectin levels were diminished to control (IgG) levels. First, we determined the effect of neutralizing TNF-α antibodies, at doses of 0.05 and 0.5 μg/ml, on the upregulation of fibronectin synthesis in the presence of exogenous TNF-α, at doses of 5 and 10 ng/ml (n = six experiments). Neutralization of TNF-α activity at a dose of 5 ng/ml with TNF-α antibodies at a dose of 0.5 μg/ml decreased the upregulation of fibronectin to control (IgG) levels ($P < 0.05$), and there was a trend with TNF-α antibodies at a dose of 0.05 μg/ml ($P < 0.1$). At a dose of 10 ng/ml, exogenous TNF-α upregulation of fibronectin synthesis was also significantly reduced to control (IgG) levels at both 0.05 and 0.5 μg/ml ($P < 0.05$) concentrations of TNF-α antibodies (Fig. 5). Similarly, neutralization of 100 ng/ml exogenous IL-1β with IL-1β antibodies at a 1:50 dilution significantly downregulated the increase in fibronectin levels ($P < 0.05$) to control (IgG) values (Fig. 6) (n = four experiments).

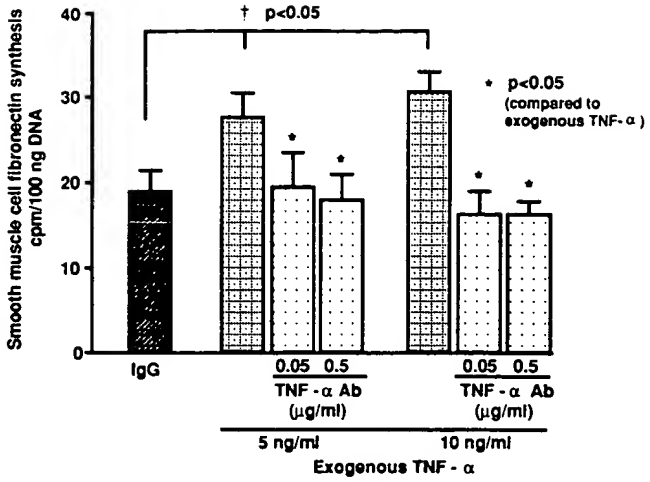


Fig. 5. Effect of TNF-α neutralizing antibodies on fibronectin levels in TNF-α-stimulated normal coronary artery smooth muscle cells. Neutralization of TNF-α activity by TNF-α antibodies decreased the upregulated levels of fibronectin in coronary artery smooth muscle cells to control (IgG) values. A dose of 0.05 μg/ml of TNF-α antibodies (Ab) showed a trend in downregulating fibronectin in the presence of 5 ng/ml of exogenous TNF-α ($P < 0.1$), while a dose of 0.5 μg/ml had a significant effect ($P < 0.05$). Nevertheless, TNF-α antibodies at both doses of 0.05 and 0.5 μg/ml significantly decreased fibronectin levels in the presence of 10 ng/ml of exogenous TNF-α ($P < 0.05$). Values depicted in the graph represent mean values ± SE from six different experiments.

Exogenous TNF-α upregulation of normal coronary artery smooth muscle cell fibronectin synthesis via IL-1β

Since TNF-α is known to induce IL-1β synthesis in many cell types, we addressed the possibility that TNF-α upregulated fibronectin via induction of IL-1β. Normal coronary artery smooth muscle cell fibronectin synthesis was assessed following stimulation with exogenous TNF-α at doses of 5 and 10 ng/ml, but in the presence of IL-1β antibodies. Indeed, TNF-α stimulation of smooth muscle cell fibronectin production at doses of 5 and 10 ng/ml was abrogated by a 1:50 concentration of IL-1β antibodies ($P < 0.05$) (n = three exper-

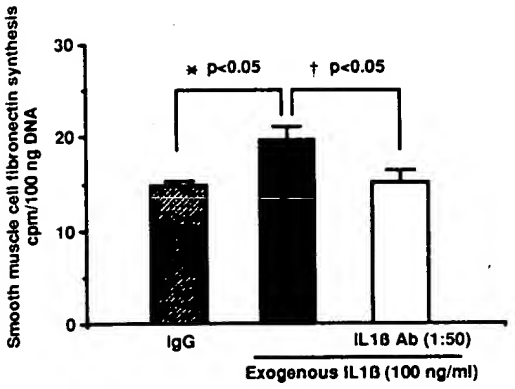


Fig. 6. Effect of IL-1β neutralizing antibodies on fibronectin levels in IL-1β-stimulated normal coronary artery smooth muscle cells. Neutralization of IL-1β activity by IL-1β antibodies (Ab) decreased the upregulated levels of fibronectin in coronary artery smooth muscle cells. Enhanced levels of fibronectin observed with exogenous IL-1β at the dose of 100 ng/ml ($P < 0.05$) were decreased to control (IgG) levels in the presence of IL-1β antibodies at a 1:50 dilution ($P < 0.05$). Values depicted in the graph represent mean values ± SE from four different experiments.

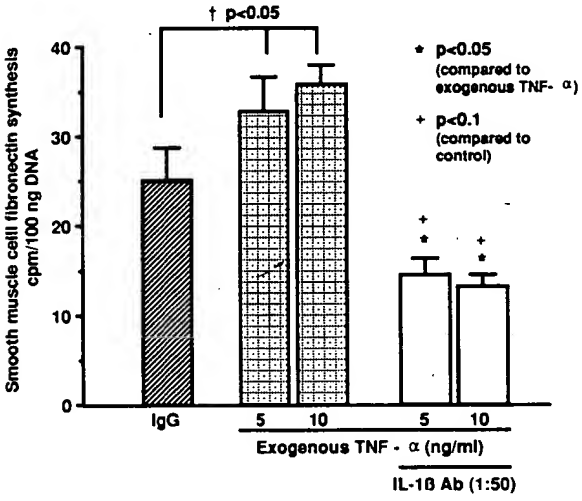


Fig. 7. Effect of IL-1β antibodies on TNF-α-stimulated synthesis of fibronectin. The exogenous TNF-α increase in fibronectin levels compared to control ($P < 0.05$), at concentrations of 5 and 10 ng/ml, was downregulated when IL-1β antibodies (Ab) were added at a dilution of 1:50 ($P < 0.05$, respectively). Notably, fibronectin production decreased to below control (IgG) levels in the presence of IL-1β antibodies trended toward significance ($P < 0.1$). Values depicted in the graph represent mean values ± SE from three different experiments.

iments) (Fig. 7). A similar response to TNF-α-stimulated fibronectin synthesis in the presence of IL-1β antibodies was observed in host coronary artery smooth muscle cells (data not shown). Interestingly, reduction of fibronectin synthesis by IL-1β antibodies below control (IgG) levels trended toward significance ($P < 0.1$).

Exogenous IL-1β upregulation of normal coronary artery smooth muscle cell fibronectin synthesis via TNF-α

Having established that exogenous TNF-α increases fibronectin synthesis by an effect which appears to be

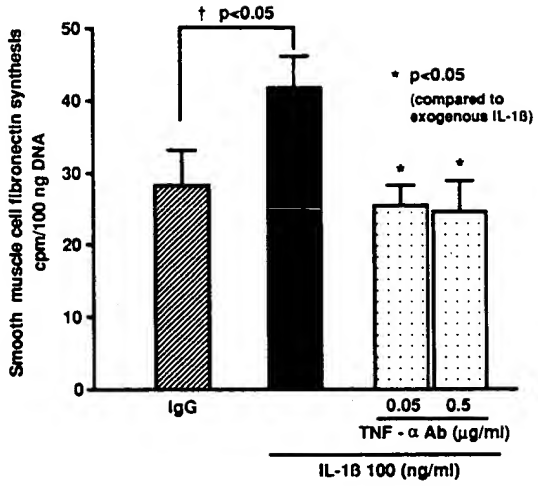


Fig. 8. IL-1β-stimulated fibronectin synthesis in the presence of neutralizing TNF-α antibodies. Interleukin-1β at a concentration of 100 ng/ml simulated fibronectin, but this effect was abrogated in the presence of neutralizing antibodies (Ab) to TNF-α in concentrations of 0.05 and 0.5 µg/ml ($P < 0.05$), lowering fibronectin production to control (IgG) values. Values depicted in the graph represent mean values ± SE from six different experiments.

mediated by IL-1β, we next investigated whether there could also be a reciprocal upregulation of fibronectin synthesis by IL-1β via TNF-α. We, thus, determined the effect of neutralizing TNF-α antibodies on IL-1β-induced smooth muscle cell fibronectin production. In fact, IL-1β stimulation of fibronectin synthesis, at a dose of 100 ng/ml, could be abrogated with TNF-α antibodies at doses of 0.05 and 0.5 µg/ml ($P < 0.05$) (n = six experiments) (Fig. 8). This downregulation to control (IgG) levels differs from previous studies in which TNF-α-stimulated fibronectin synthesis was reduced by IL-1β antibodies to below control (IgG) levels.

Effect of fibronectin synthesis induced by both IL-1β and TNF-α in normal coronary artery smooth muscle cells

Since IL-1β and TNF-α were previously shown to have synergistic effect in stimulating specific cellular functions (Caldwell and Emerson, 1994; Varani et al., 1989) and since they individually upregulated fibronectin production in coronary artery smooth muscle cells, we investigated a potential synergism of these cytokines in our system. Hence, we measured fibronectin synthesis in the presence of high dose for both cytokines, i.e. IL-1β (100 ng/ml) and TNF-α (5 and 10 ng/ml), which were proven to be the most effective in our system. Indeed, we observed a three to fourfold increase in fibronectin levels compared to control ($P < 0.05$), and a 30 to 50% increase compared to fibronectin levels with either IL-1β or TNF-α alone ($P < 0.05$) (Fig. 9) (n = three experiments). These studies were performed with control IgG added to all samples in the presence of exogenous cytokines, in order to rule out a possible effect that our control immunoglobulin could have in the net synthesis of fibronectin. Absolute values were essentially the same in the absence of IgG in all samples treated with either cytokine alone or in combina-

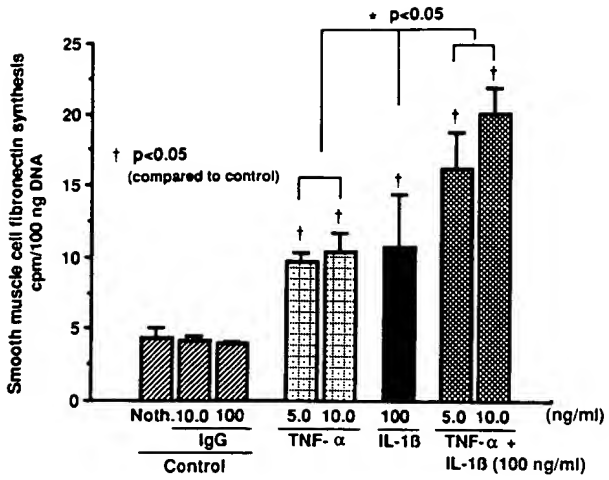


Fig. 9. Synergistic effect of both IL-1β and TNF-α in the synthesis of fibronectin. The combination of both IL-1β, at a dose of 100 ng/ml, and TNF-α, at doses of 5 and 10 ng/ml, caused a 30 to 50% increase in fibronectin levels compared to either cytokine alone ($P < 0.05$), which also had a stimulatory effect on fibronectin production from basal levels (control: no treatment (no IgG), normal rabbit IgG (10 and 100 ng/ml) ($P < 0.05$). Normal rabbit IgG was added to the cytokine-treated samples, at the concentration of 10 ng/ml for IL-1β-treated samples and 10 ng/ml for TNF-α-treated samples. The values depicted in the graph are essentially similar to those observed when control IgG was not added to the cytokine-treated samples, either alone or in combination (data not shown). Values are representative of three different experiments.

tion (data not shown). Additional control samples with IgG were compared with samples which were not treated and the values were fundamentally the same (Fig. 9), in keeping with previous experiments performed in our laboratory.

DISCUSSION

In the current study, we have demonstrated, for the first time, a reciprocal interrelation between IL-1β and TNF-α in modulating fibronectin, a major extracellular matrix protein and component of the basement membrane. Our studies using an allograft cardiac transplant model further confirmed an association between increased donor coronary artery smooth muscle cell fibronectin synthesis and co-induction of cytokine activity in the development of graft arteriopathy. Tumor necrosis factor-α appears to have the same modulatory influence on fibronectin synthesis previously shown with IL-1β. The increase in donor coronary artery smooth muscle cell fibronectin synthesis is neutralized by TNF-α antibodies and host coronary artery smooth muscle cell fibronectin synthesis can be induced by exogenous TNF-α. Moreover, there is an interdependence between exogenous IL-1β and TNF-α upregulation of fibronectin synthesis in normal coronary artery smooth muscle cells. Interleukin-1β antibodies prevent TNF-α stimulation of fibronectin, downregulating fibronectin below control levels, and TNF-α antibodies decrease the upregulation of fibronectin synthesis induced by IL-1β. In addition, both cytokines exert a greater, although not additive, effect in enhancing fibronectin levels when compared to either cytokine alone. These findings support their interdependence and an IL-1β-TNF-α 'cytokine loop' involved in the upregulation of synthesis of the extracellular matrix protein fibronectin and is likely of pathophysiologic significance in the neointimal formation seen in graft arteriopathy. The latter is corroborated by studies in which TNF-α blockade reduced IL-1β expression and the development of graft arteriopathy in rabbits following heterotopic cardiac transplantation (Clausell et al., 1994).

Our initial in vivo studies using coronary arteries from piglets following cardiac transplantation revealed the presence of an immune-inflammatory reaction in the vessel wall characterized by positive immunostaining for inflammatory cells, expression of MHC II antigens and accumulation of IL-1β (Clausell et al., 1993). We have, in this study, specifically addressed the expression of TNF-α in the allograft coronary arteries and we were able to show increased immunostaining in donor vessels compared to host vessels. The intense immunostaining in donor vessels was observed on endothelial cells with or without inflammatory cells attached to the endothelium, and also in smooth muscle cells. The presence of TNF-α has also been demonstrated in different organ transplants both at the protein and mRNA levels (Krams et al., 1992; Noronha et al., 1992; Vandenbroeke et al., 1991). In fact, in biopsy specimens from human cardiac transplants, TNF-α was detected by immunohistochemistry, and its expression appeared to be related to the degree of myocardial rejection as it was closely associated with the number of infiltrating inflammatory cells (Arbustini et al., 1991). Also, high circulating levels of TNF-α have been correlated with poor outcome of patients following liver transplantation (Imagawa et al., 1990).

While Mauviel and colleagues (1988) reported downregulation of fibronectin by TNF-α in human fibroblats, other studies demonstrated that this cytokine upregulates fibronectin synthesis in normal melanocytes and malignant melanoma cells (Varani et al., 1989). This latter study suggested a different cytokine interaction by showing induction of fibronectin by TNF-α following addition of IFN-γ (Varani et al., 1989). The differences in these studies could be attributed to the cell types studied, and the experimental conditions. There may also be upregulation of TNF-α by fibronectin as demonstrated, recently, in studies where macrophages and $CD4^+$ T cells plated on fibronectin enriched matrices induced synthesis of TNF-α (Hershkoviz et al., 1993).

In our study, we have related smooth muscle cell fibronectin synthesis to an IL-1β-TNF-α 'cytokine loop' in the setting of allograft arteriopathy. We have previously described the effects of IL-1β on host and donor coronary artery smooth muscle cell fibronectin synthesis, and this appeared to be associated with increased endogenously synthesized IL-1β by smooth muscle cells (Clausell and Rabinovitch, 1993). These experiments have shown, however, that TNF-α also has a similar effect on donor smooth muscle cells likely due to increased endogenous production of this cytokine, as demonstrated in donor coronary arteries in vivo. Downregulation of donor fibronectin levels were observed

when TNF-α neutralizing antibodies were used, bringing fibronectin synthesis to levels observed in host cells where blockade of TNF-α activity had no effect. Thus, it appears that the effect of fibronectin synthesis with neutralization of TNF-α activity may also depend on the endogenous level of TNF-α or on the level of fibronectin synthesis. Although our experiments using host and donor tissues could only be performed in two animals, due to limited availability of tissue for culturing vascular cells, we have extensively demonstrated upregulation of fibronectin synthesis in donor smooth muscle cells compared to host, and both the neutralization of this effect by IL-1β antibodies in donor cells and a stimulatory effect using exogenous IL-1β in host cells (Clausell and Rabinovitch, 1993).

It is known that IL-1 and TNF-α share many biological activities, in addition to the fact that they can be reciprocally co-induced (Bethea et al., 1992; Nawroth et al., 1986). Moreover, studies on intra-graft events related to cytokine mechanisms indicated that increase in TNF-α expression in grafts occurs very early in the course of activation of the 'cytokine cascade' preceding the expression of IL-1 (Ford et al., 1990). Also, endothelial cell induction of IL-1 was observed upon stimulation with TNF-α (Nawroth et al., 1986). Using normal porcine coronary artery smooth muscle cells, we observed upregulation of fibronectin levels upon stimulation with TNF-α, albeit not to the extent seen with host cells, which may reflect the different milieu of cytokines and growth factors to which the host cells were subjected in the transplant model. Furthermore, although the levels of fibronectin synthesis may differ in each experiment, they consistently reflected either the stimulatory activity of exogenous cytokine or the inhibitory effect of neutralizing antibodies compared to basal production in a steady state.

The effect of TNF-α on fibronectin production in normal coronary artery smooth muscle cells could be overcome by the addition of IL-1β neutralizing antibodies. Blockade of IL-1β upon stimulation of vascular smooth muscle cells decreased fibronectin production below basal levels, although this effect could not be seen when smooth muscle cell fibronectin synthesis had not been primed first by TNF-α. We previously observed that IL-1β antibodies could decrease fibronectin synthesis in donor smooth muscle cells with high endogenous levels of IL-1β but not in host levels, with low endogenous levels of IL-1β. It appears, therefore, that the capacity of IL-1β to regulate intracellular mechanisms controlling fibronectin production may depend on a specific level of expression of either the cytokine or its receptors and this may be a function of a particular cell phenotype. These data taken together, in addition to our previous work related to IL-1β regulation of fibronectin synthesis (Clausell and Rabinovitch, 1993), implicate this cytokine as a key mediator of matrix production in response to TNF-α.

We further investigated whether the IL-1β effect in enhancing fibronectin production could be mediated by TNF-α, sinc IL-1β has been shown to induce TNF-α gene expression by activation of NF-κB via a protein kinase-C-dependent pathway (Bethea et al., 1992). Our previous studies revealed that exogenous IL-1β increased basal levels of fibronectin in host coronary artery smooth muscle cells and, in this study, we demonstrated that the same effect using normal coronary artery smooth muscle cells could be overcome by the addition of IL-1β neutralizing antibodies. Tumor necrosis factor-α neutralizing antibodies decreased fibronectin synthesis to basal (control) levels when IL-1β was used to stimulate coronary artery smooth muscle cells, suggesting that the effect of IL-1β could be due to induction of TNF-α expression. Neutralizing TNF-α activity did not affect basal production of fibronectin, since control levels were not affected by TNF-α antibodies.

Previous studies suggest that the molecular mechanisms of IL-1β regulation of fibronectin is complex, but associated with increased mRNA levels and transcription rates (Clausell and Rabinovitch, 1993; Hu et al., 1993). These findings imply that IL-1β and TNF-α upregulate fibronectin through co-dependent intracellular signaling pathways. The mechanism may also involve upregulation of IL-1 receptors, as TNF-α has been shown to upregulate IL-1 receptors (Bry et al., 1993). If TNF-α induces IL-1 and IL-1 receptors and IL-1 induces TNF-α, then IL-1 antibodies, in contrast to TNF-α antibodies, would leave the TNF-α-stimulated IL-1 receptors unoccupied and this might result in a negative feedback loop accounting for the tendency for fibronectin levels to fall below basal values.

Finally, we observed a further increase (30 to 50%) in fibronectin synthesis when both IL-1β and TNF-α were added simultaneously to normal coronary artery smooth muscle cells. That an additive effect of IL-1β and TNF-α upregulation of fibronectin levels was not seen, supports the interdependence of the two cytokines related to a final column pathway. Alternatively, it may represent a saturated state of production of this matrix protein, although recent unpublished studies from our laboratory would not support saturation of cytokine-mediated effect at the doses used. There are examples in the literature of combined and synergistic effects of cytokines in regulating cellular functions. Caldwell and Emerson (1994) showed that IL-1α and TNF-α had greater than additive effects on the production of myeloid colony-stimulating factors by human bone marrow and cloned stromal cells. Kobayashi and colleagues (1990), on the other hand, did not show an additive or synergistic effect of IL-1β and TNF-α in decreasing the synthesis of glycosaminoglycans in porcine aortic endothelial cells.

In summary, our results indicate that there is a 'cytokine loop' whereby increased expression of TNF-α and IL-1β activity in allograft coronary arteries are reciprocally induced and mediate the upregulation of fibronectin synthesis by vascular smooth muscle cells, a feature we have previously associated with the development of graft arteriopathy. Based on these observations, we speculate that the interplay between TNF-α and IL-1β in vivo, co-inducing their expression, leads to increased synthesis of fibronectin in coronary artery smooth muscle cells with subsequent accumulation in the subendothelial space. The functional significance of the accumulation of this matrix protein could be related to two distinct mechanisms of vascular pathobiology. Increased fibronectin may induce smooth muscle migration into the subendothelial space (Boudreau et al.,

1991; Linask and Lash, 1988); but it may also increase the migration and trafficking of immune-reactive cells into the vessel wall, as we have recently demonstrated in vitro (Molossi et al., 1994).

ACKNOWLEDGMENTS

The authors are most grateful to Dr. Roberto Diaz for performing the piglet cardiac transplants and to Claire Coulber for expert technical assistance and advice. We also thank Joan Jowlabar and Susy Taylor for the graphic art work and secretarial assistance, Mike Starr for assistance in preparing the colour plates, and Dr. Brian McCrindle for his counsel with regards to the statistical analyses. The authors are indebted with Dr. N. Staite, from Upjohn laboratories, who has given the extensive supply of recombinant human interleukin-1β.

Supported by a Program grant PG12351 from the Medical Research Council of Canada. Dr. S. Molossi is a Clinician Scientist from the Lunenfeld Foundation, Research Institute, The Hospital For Sick Children, and is also supported by the Heart and Stroke Foundation of Canada. Dr. N. Clausell was supported by Conselho Nacional de Apoio a Pesquisa (CAPES), Brazil. Dr. M. Rabinovitch is a Career Investigator of the Heart and Stroke Foundation of Ontario.

LITERATURE CITED

Arbustini, E., Grasso, M., Diegoli, M., Bramerio, M., Foglieni, A., Albertario, M., Martinelli, L., Gavazzi, A., Goggi, C., Campana, C., and Vigano, M. (1991) Expression of tumor necrosis factor in human acute cardiac rejection. Am. J. Pathol. *139*:709–715.

Bethea, J., Gillespie, G., and Benveniste, E. (1992) Interleukin-1β induction of TNF-α gene expression: Involvement of protein kinase C. J. Cell Physiol., *152*:264–273.

Boudreau, N., and Rabinovitch, M. (1991) Developmentally regulated changes in extracellular matrix in endothelial and smooth muscle cells in the ductus arteriosus may be related to intimal proliferation. Lab. Invest., *64*:187–199.

Boudreau, N., Turley, E., and Rabinovitch, M. (1991) Fibronectin, hyaluronan and a hyaluronan binding protein contribute to increased migration of ductus arteriosus smooth muscle cell. Dev. Biol., *143*:235–247.

Bry, K., Lappalainen, U., and Hallman, M. (1993) Interleukin-1 binding and prostaglandin E_2 synthesis by amnion cells in culture: Regulation by tumor necrosis factor-α, transforming growth factor-β, and interleukin-1 receptor antagonist. Biochim. Biophys. Acta, *1181*:31–36.

Caldwell, J., and Emerson, S.G. (1994) IL-1α and TNF-α act synergistically to stimulate production of myeloid colony-stimulating factors by cultured human bone marrow stromal cells and clonal stromal cell strains. J. Cell Physiol., *159*:221–228.

Clausell, N., and Rabinovitch, M. (1993) Upregulation of fibronectin synthesis by interleukin-1β in coronary artery smooth muscle cells is associated with the development of the post-cardiac transplant arteriopathy in piglets. J. Clin. Invest., *92*:1850–1858.

Clausell, N., Molossi, S., and Rabinovitch, M. (1993) Increased interleukin-1β and fibronectin expression are early features of the development of the post-cardiac transplant coronary arteriopathy in piglets. Am. J. Pathol., *142*:1772–1786.

Clausell, N., Molossi, S., Sett, S., and Rabinovitch, M (1994) In vivo blockade of tumor necrosis factor-α in cholesterol-fed rabbits following cardiac transplant inhibits acute coronary artery neointimal formation. Circulation, *89*:2768–2779.

Dinarello, C. (1991) Interleukin 1 and interleukin-1 antagonism. Blood, *77*:1627–1652.

Dinarello, C., Cannon, J., Wolff, S., Bernheim, H., Beutler, B., Cerami, A., Figari, I., Palladino Jr., M., and O'Connor, J. (1986) Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med., *163*:1433–1450.

Fanslow, W., Sims, J., Sassenfeld, H., Morrissey, P., Gillis, S., Dower, S., and Widmer, M. (1991) Regulation of alloreactivity in vivo by a soluble form of interleukin-1 receptor. Science, *218*:639–742.

Ford, H., Hoffman, R., Wing, E., Magee, D., McIntyre, L., and Simmons, R. (1990) Tumor necrosis factor, macrophage colony-stimulating factor, and interleukin 1 production within sponge matrix allografts. Transplantation, *50*:460–466.

Gao, S., Hunt, S., and Schroeder, J. (1990) Accelerated transplant coronary artery disease. Semin. Thorac. Cardiovasc. Surg., *2*:241.

Gorospe, M., Kumar, S., and Baglioni, C. (1993) Tumor necrosis factor increases stability of interleukin-1 mRNA by activating protein kinase C. J. Biol. Chem., *268*:6214–6220.

Gown, A., Tsukada, T., and Ross, R. (1986) Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. Am. J. Pathol., *125*:191–207.

Hancock, W., Tanaka, K., Salem, H., Tilney, N., Atkins, R., and Kupiec-Weglinski, J., (1991) TNF as a mediator of cardiac transplant rejection, including effects on the intra-graft protein C/protein S/thrombomodulin pathway. Transplant. Proc., *23*:235–237.

Hershkoviz, R., Gilat, D., Miron, S., Mekori, Y., Aderka, D., Wallach, D., Vlodavsky, I., Cohen, I., and Lider, O. (1993) Extracellular matrix induces tumor necrosis factor-α secretion by an interaction between resting rat $CD4^+$ T cells and macrophages. Immunology, *78*:50–57.

Hu, W., Coulber, C., and Rabinovitch, M. (1993) Transcriptional regulation of fibronectin by interleukin-1β related to accelerated graft atherosclerosis following cardiac transplant. Circulation [suppl part II], *88*:920.

Imagawa, D., Millis, J., Olthoff, K., Derus, L., Chia, D., Sugich, L., Ozawa, M. Dempsey, R., Iwaki, Y., Levy, P., Terasaki, P., and Busuttil, R. (1990) The role of tumor necrosis factor in allograft rejection. I—Evidence that elevated levels of tumor necrosis factor-alfa predict rejection following orthotopic liver transplantation. Transplantation, *50*:219–225.

Imagawa, D., Millis, J., Seu, P., Olthoff, K., Hart, J., Wasef, E., Dempsey, R., Stephens, S., and Busuttil, R. (1991) The role of tumor necrosis factor in allograft rejection. III—Evidence that anti-TNF antibody prolongs allograft survival in rats with acute rejection. Transplantation, *51*:57–62.

Kaji, T., Hiraga, S., Yamamoto, C., Sakamoto, M., Nakashima, Y., Sueishi, K., and Koizumi, F. (1993) Tumor necrosis factor-alpha-induced alteration of glycosaminoglycans in cultured vascular smooth-muscle cells. Biochim. Biophys. Acta, *1176*:20–26.

Kobayashi, M., Shimada, K., and Ozawa, T. (1990) Human recombinant interleukin-1β and tumor necrosis factor-α-mediated suppression of heparin-like compounds on cultured porcine aortic endothelial cells. J Cell Physiol *144*:383–390.

Koike, K., Hesslein, P., Dasmahapatra, H., Wilson, G., Finlay, C., David, S., Kielmanowicz, S., and Coles, J. (1988) Telemetric detection of cardiac allograft rejection. Correlation of electrophysiological, histological, and biochemical changes during unmodified rejection. Circulation, *78* [suppl I]:I106–I112.

Krams, S., Falco, D., Villanueva, J., Rabkin, J., Tomlanovich, S., Vincenti, F., Amend, W., Melzer, J., Garovoy, M., Roberts, J., Ascher, N., and Martinez, O. (1992) Cytokine and T cell receptor gene expression at the site of allograft rejection. Transplantation, *53*:151–156.

Kruppa, G., Thoma, B., Machleidt, T., Wiegmann, K., and Kronke, M. (1992) Inhibition of tumor necrosis factor (TNF)-mediated NF-κB activation by selective blockade of the human 55-kDa TNF receptor. J. Immunol., *148*:3152–3157.

Le, J., and Vilcek, J. (1987) Tumor necrosis factor and interleukin-1: Cytokines with multiple overlapping biological activities. Lab. Invest., *56*:234–248.

Le Pecq, J. (1971) Use of ethidium bromide for separation and determination of nucleic acids for various conformational forms and measurement of their associated enzymes. In: Methods of Biochemical Analysis. D. Glick (ed) New York, John Wiley & Sons.

Libby, P., Salomon, R., Payne, D., Schoen, F., and Pober, J. (1989) Functions of vascular wall cells related to development of transplantation-associated coronary arteriosclerosis. Transplant. Proc., *21*:3677–3684.

Linask, K., and Lash, J. (1988) A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. Dev. Biol., *129*:315–323.

Mathison, J., Wolfson, E., and Ulevitch, R. (1988) Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. J. Clin. Invest., *81*:1925–1937.

Mauviel, A., Daireaux, M., Redini, F., Galera, P., Loyau, G., and Pujol, J. (1988) Tumor necrosis factor inhibits collagen and fibronectin synthesis in human dermal fibroblasts. FEBS Lett., *236*:47–52.

Molossi, S., Clausell, N., and Rabinovitch, M. (1993) Coronary artery endothelial interleukin-1β mediates enhanced fibronectin produc-

tion related to post-cardiac transplant arteriopathy in piglets. Circulation, *88*[Part II]:248–256.

Molossi, S., Elices, M., and Rabinovitch, M. (1994) Blockade of interleukin-1β-induced fibronectin/lymphocyte interaction in vitro inhibits lymphocyte transendothelial migration. FASEB J., *8*:A1018 (abstract).

Nawroth, P., Bank, I., Handley, D., Cassimeris, J., Chess, L., and Stern, D. (1986) Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J. Exp. Med., *163*:1363–1375.

Noronha, I., Eberlein-Gonska, M., Hartley, B., Stephens, S., Cameron, J., and Waldherr, R. (1992) In situ expression of tumor necrosis factor-alpha, interferon-gamma, and interleukin-2 receptors in renal allograft biopsies. Transplantation, *54*:1017–1024.

Piguet, P., Grau, G., Vesin, C., Loetscher, H., Gentz, R., and Lesslauer, W. (1992) Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. Immunology, *77*:510–514.

Ross, R. (1971) The smooth muscle cell II. Growth of smooth muscle in culture and formation of elastic fibres. J. Cell. Biol., *50*:172–186.

Saklatvala, J., Sarsfield, S., and Townsend, Y. (1985) Pig interleukin 1. Purification of two immunologically different leukocyte proteins that cause cartilage resorption, lymphocyte activation and fever. J. Exp. Med., *162*:1208–1222.

Salomon, R., Hughes, C., Schoen, F., Payne, D., Pober, J., and Libby, P. (1991) Human coronary transplantation-associated arteriosclerosis. Am. J. Pathol., *138*:791–798.

Uretsky, B.F., Murali, S., Reddy, P.S., Rabin, B., Lee, A., Griffith, B.P., Hardesty, R.L., Trento, A., and Bahnson, H.T. (1987) Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporine and prednisone. Circulation, *76*:827–834.

Vandenbroeke, C., Caillet-Zucman, S., Legendre, C., Noel, H., Kreis, H., Wooddrow, D., Bach, J.-F., and Tovey, M. (1991) Differential in situ expression of cytokines in renal allograft rejection. Transplantation, *51*:601–609.

Varani, J., Mitra, R., McClenic, B., Fligiel, S., Inman, D., Dixit, Y., and Nickoloff, B. (1989) Modulation of fibronectin production in normal human melanocytes and malignant melanoma cells by interferon-γ and tumor necrosis factor-α. Am. J. Pathol., *134*:827–836.

Warner, S., Auger, K., and Libby, P. (1987) Interleukin 1 induces Interleukin 1 II. Recombinant human interleukin 1 induces interleukin 1 production by adult human vascular endothelial cells. J. Immunol., *139*:1911–1917.

Wrana, J., Maeno, M., Hawrylyshyn, B., Yao, K., Domenicucci, C., and Sodek, J. (1988) Differential effects of transforming growth factor beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell population. J. Cell Biol., *106*:915–924.

STIC-ILL DL 4372695 Address No

363813

Fr m: Canella, Karen
Sent: Sunday, September 16, 2001 6:01 PM
To: STIC-ILL
Subject: ill order 08/602,272

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 08/602,272

1. Asaio Journal, 1994 Jul-Sep, 40(3):M554-M559
2. Gastroenterology, 1995, Vol. 108, No. 4, suppl., p. A838
3. Israel Journal of Medical Sciences, 1991 Jan, 27(1):52-60
4. J of Interferon and Cytokine Research, 1995 Sep, 15(9):819-825
5. Arteriosclerosis, Thrombosis and Vascular Biology, 1995 Feb, 15(2):258-268
6. J of Cellular Physiology, 1995 Apr, 163(1):19-29
7. Blood, 1994 Jul 15, 84(2):483-489
8. Acta Anaesthesiologica Scandinavia. Supplementum, 1991, Vol. 95, pp. 40-54
9. Circulation, 1994, Vol. 90, No. 4, part 2, pp. I 522
10. Thromb Res, 1996 Feb 1, 81(3):315-326
11. Tumori, 1996 Jan, 82(1):78-80
12. Cytokine, 1995 Jan, 7(1):15-25
13. Crit Care Med, 1993 Mar, 21(3):318-327
14. Thromb Haemost, 1993 Feb 1, 69(2):164-172
15. Lab Invest, 1988 Apr, 58(4):365-378

PMID 1927227

Acta Anaesthesiol Scand 1991: 35, Supplementum 95: 40–54

Hypoxic pulmonary vasoconstriction in the adult respiratory distress syndrome

ÅSE JOLIN and LARS BJERTNÆS

Department of Anesthesiology, Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway

Increased pulmonary vascular resistance (PVR) and microvascular hyperpermeability resulting in lung edema and arterial hypoxemia are mainstays in the development of adult respiratory distress syndrome (ARDS). The proposed pathophysiologic mechanisms include activation of complement and polymorphonuclear leukocytes secreting lysozomal enzymes, toxic oxygen metabolites (TOM) and eicosanoids. Platelets and coagulation factors are also involved, and in the most severe cases even monocytes are activated as reflected in release of thromboplastin. The latter may elicit disseminated intravascular coagulation (DIC).
Under physiologic conditions lung blood flow is diverted from poorly to better oxygenated areas by way of hypoxic pulmonary vasoconstriction (HPV) thereby counteracting a decrease in arterial oxygenation. Many vasoactive substances have been proposed and again refuted as possible mediators of HPV. In this study we have focused on the following: histamine, catecholamines, arachidonates, calcium, phosphoinositides and TOM as well as endothelium-derived relaxing and constricting factors. Whether HPV is present in ARDS and whether it is advantageous or not seems to depend on the stage and extent of disease.
We discuss possible interactions between HPV and ARDS mediators and between HPV and various vasoactive agents tested for therapeutic effects. Out of the abundance of mediators released, prostacyclin, prostaglandin E_1, activated complement and platelet activating factor have been shown explicitly to inhibit HPV whereas others are suspected of doing so. In therapeutical use, prostacyclin has proved to reduce PVR and at the same time enhance cardiac output and oxygen delivery. In mild to moderate ARDS, improvement of arterial oxygenation has also been obtained employing almitrine bismesylate, a potentiator of HPV. Experimentally, adenosine effectively reduces increments in PVR and microvascular permeability with modest effects on systemic circulation. However, further investigations are warranted to decide whether adenosine or more specific blockers as, for instance, monoclonal antibodies against tumor necrosis factor should be integrated in ARDS therapy in the future.

Key words: Adult respiratory distress syndrome; Arachidonates; Eicosanoids; Hypoxic pulmonary vasoconstriction; Phosphoinositides; Pulmonary vascular resistance; Toxic oxygen metabolites.

Severe arterial hypoxemia is one of the hallmarks of adult respiratory distress syndrome (ARDS). Although a myriad of diseases may be associated with ARDS, the acute lung function abnormalities have a common denominator: a microvascular hyperpermeability leading to a progressive accumulation of extravascular water in the lung. Deterioration of the endothelial and epithelial cell linings leads to flooding confined to the interstitial space and alveoli (1). The resulting gas exchange abnormalities are mainly caused by intrapulmonary right-to-left shunts and by ventilation/perfusion inequalities.

Under physiologic conditions, the deleterious effects of poorly ventilated lung regions on arterial oxygenation are counteracted by hypoxic pulmonary vasoconstriction (HPV) which redistributes blood towards more amply ventilated areas (2). Out of the abundance of reports dealing with human ARDS, no one has focused particularly on HPV and its possible interaction with mediators of ARDS. High altitude pulmonary edema is a clinical and pathophysiologic entity involving HPV, which has many features in common with ARDS, though no direct relationship has as yet been documented (3).

Our purpose here is first to survey vasoactive substances considered to participate in the mediation of HPV. Secondly, we want to assess whether HPV is influenced by changes in pulmonary vascular tone associated with ARDS and whether this effect is favorable or detrimental. Thirdly, we will discuss how vasoactive agents that have been tested for effects on patients with ARDS might interact with HPV.

MEDIATION OF HPV

General considerations

HPV has been a most puzzling challenge to investigators for more than 40 years. The thoughts we present represent our personal view and should not be understood as a generally accepted concept.

For various reasons it is difficult to assess the effect of drugs on HPV. First, because it varies between individuals of the same species as well as between different species (4); secondly, because it depends both on the experimental or clinical situation as well as on the strength and duration of stimulation. In studies carried out in man or intact animals it is, for example, of importance whether hypoxia has been confined to one lung, a lobe of a lung or to both lungs. The last of these three methods of inducing hypoxia may cause changes in pulmonary hemodynamics due both to intrapulmonary and systemic influences (nervous or humoral), thereby making results difficult to interpret. Investigators employing isolated lungs, on the other hand, have excluded the possibility of systemically induced influences. An advantage of using isolated lungs is that pharmacological substances can be administered in higher doses than in intact animals. Some drug receptors are completely blocked only at relatively high concentrations of the antagonist.

While earlier workers believed that HPV is elicited by a single mediator responding to stimulation of an hypoxia-sensitive area remote from vascular smooth muscle, current researchers favor the hypothesis that "multiple factors differing in combination in different species" are at play (5). Response activation might include both constricting and dilating stimuli. This view is supported by the observation that the lung vasculature may dilate in response to hypoxia when the possibility to constrict is blocked (6). As yet, neither the hypoxia-sensitive site nor the chain of mediators being involved in vasoconstriction have been fully understood. A further point to consider is whether the drug tested for an effect on HPV acts specifically, i.e. by interfering with a mediator or chain of mediators leading to vascular smooth muscle contraction, or by a paralyzing effect on vascular smooth muscle. A distinction between these two possibilities can be made by parallel demonstration of unaltered vasoconstrictor responses to other stimuli at concentrations of the test substance that abolish HPV (7). Fig. 1 is an attempt to present a unifying hypothesis of substances suggestedly involved in the mediation of hypoxia-induced constriction of lung vessels and some of their blockers and potentiators.

Histamine

A commonly held opinion a few years ago was that endogenously administered histamine mediates HPV (8, 9). Thus, in the rat isolated lung, large doses of antihistamines and histamine-depleters reduce HPV, whereas semicarbazide (a histaminase inhibitor) potentiates it. Histamine concentration in lung effluent blood has been reported to increase during hypoxia (10). In addition, deposits of histamine contained in mast cells are strategically placed along the course of the pulmonary resistance vessels (11). However, results obtained by various investigators have been conflicting, probably due to differences both in species and experimental design. Although histamine is the substance most thoroughly investigated for the role as mediator of HPV, ambiguity has arisen as far as the general applicability of this concept is concerned.

Autonomic nervous system

Shortly after HPV had been described (2), Logaras (12) demonstrated in cats that HPV acts independently of the autonomic nervous system. Although some subsequent workers found reduced responses following administration of α-adrenergic blocking agents in cat (13–15), most investigators favor the view that HPV is not obtunded by these agents (16–18). In contrast, β-adrenergic agonists such as isoprenaline have proved to reduce pulmonary vascular resistence (PVR) in experimental animals under normoxic as well as hypoxic conditions (15, 19–22). On the basis of these findings it was not surprising that intravenous administration of isoprenaline post-operatively to patients undergoing cardiac surgery resulted in increased fraction of blood shunted in the lungs (23). It has furthermore been recognized that broncho-dilator treatment of patients with bronchial asthma employing isoprenaline is frequently associated with increased venous admixture (24) and enhancement of ventilation/perfusion mismatching (25). Isoprenaline exerts its action on a well defined target, the β-receptor of the vascular smooth muscle cell. Agonist binding causes activation of adenylate cyclase, producing the "second messenger", 3′,5′-cyclic adenosine monophosphate (cAMP) from ATP within the target cell. In this respect, the coupling between the β-adrenergic receptor and the enzyme adenylate cyclase is similar to the coupling between adenylate cyclase and hormone receptors to certain polypeptide hormones, such as glucagon, ACTH and secretin (26). Particularly interesting in this connection is the observation in dog that lung cAMP decreases during hypoxia-induced pulmonary vasoconstriction (27) whereas liver cAMP remains unaltered (28).

Terbutaline is a specific β-2 receptor stimulating agent which is widely used as an inhalation bronchodilator for treatment of bronchial asthma. In pigs, reduction of HPV was followed by an increase in pulmonary level of cAMP (29). Substances which increase the intracellular level of cAMP, such as glucagon and aminophylline, have also been found to reduce HPV (30, 31). Glucagon and aminophylline increase the intracellular concentration of cAMP in two

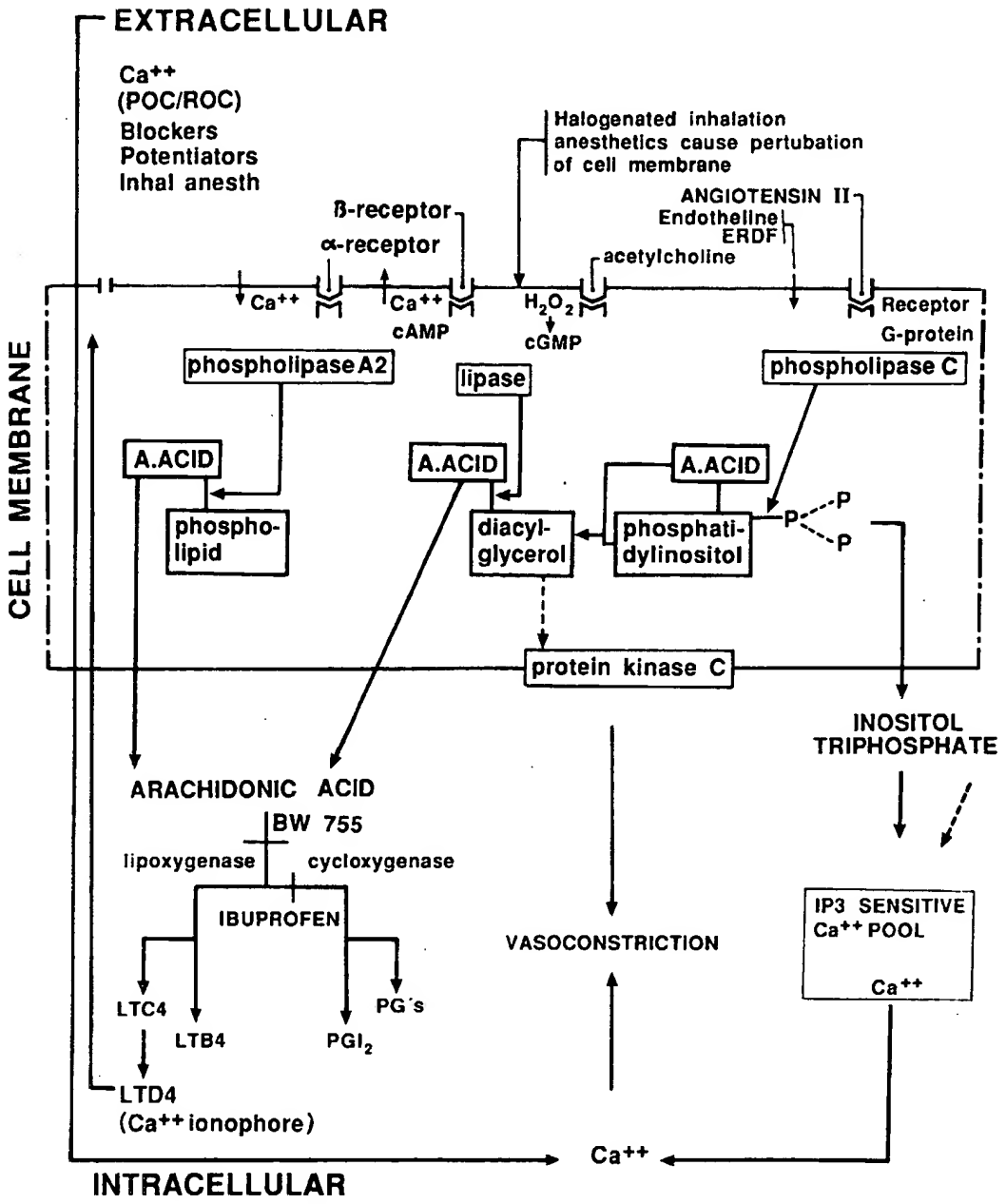


Fig. 1. Hypothesized chain of mediators of hypoxic pulmonary vasoconstriction (HPV). It is unknown how hypoxia elicits constriction, but possibly by directly affecting vascular smooth muscle of small pulmonary arteries. Arachidonic acid emanating from phospholipid and diacylglycerol (product of phosphatidyl-inositol) of the cell membrane phospholipid bilayer, may be degraded into cycloxygenase and lipoxygenase metabolites. The latter (LTC4, LTD4) may act as calciumionophores acting to enhance influx of Ca^{2+} both through potential operated and receptor operated (POC/ROC) channels (43, 46). BW 755 is a combined cycloxygenase and lipoxygenase blocker whereas ibuprofen is a blocker of the cycloxygenase pathway. Inositoltriphosphate suggestedly induces release of Ca^{2+} from sarcoplasmatic reticulum. Phosphorylation by inositoltriphosphate or diacylglycerol activated protein kinase C, may play a role in mobilization of extracellular Ca^{2+} (50). α-adrenergic agonists enhance intracellular Ca^{2+} whereas β-agonists have the opposite effect, both contributing to the basal tone of the vessels. Hydrogen peroxide suggestedly acts via enhanced 3,5′-cyclic guanosine monophosphate (cGMP) to dilate the vessel, the latter effect being antagonized by the enzyme catalase (56). Hypoxia reduces generation of endothelium derived relaxing factor (EDRF). It still remains unknown how hypoxia affects the formation of endothelin, a constrictor derived from endothelium. Angiotensin II constricts arterioles via a receptor protein (G-protein) independent of the calcium mechanism. See text for further details.

different ways: the former by increasing adenylate cyclase activity; the latter by inhibition of the enzyme phosphodiesterase, which transforms cAMP to AMP. The effect of β-receptor stimulation on vascular smooth muscle is still hypothetical. It has been shown both in turkey erythrocytes and in human erythrocytes that Ca^{2+} efflux is enhanced by β-agonists and inhibited by β-antagonists (26). Since vascular smooth muscle, in contrast to striated muscle, is dependent upon influx of Ca^{2+} from extracellular fluid for contraction, it is not surprising that smooth muscle contractility is enhanced by α-agonists and reduced by β-agonists. It is assumed that catecholamines help to set the background tone that is necessary for the pulmonary vascular smooth muscle to respond to hypoxia (5). If this assumption is correct, a varying degree of inhibition should be expected as well from α-adrenergic blockers as from β-receptor agonists.

Arachidonic acid metabolites

During the last few years much attention has been payed to the hypothesis that prostanoids are of importance for elicitation of HPV. Arachidonic acid originating from the hydrolytic action of membrane-bound phospholipase A_2 is stored in the phospholipid bilayer of the cell membrane. Derangement of arachidonic acid, which is blocked by BW 755, may be induced by several mechanisms following the cycloxygenase or lipoxygenase pathways. However, the link between airway hypoxia and the stimuli which trigger the derangement does not appear quite clear to us. It may be that toxic oxygen metabolites (TOM) induce the process by way of lipid-peroxidation (32).

In the lipoxygenase pathway the leukotriene-containing peptide is the most potent constrictor. In the cycloxygenase pathway prostacyclin is the predominant vasodilator and thromboxane A_2 the most powerful constrictor. Some years ago, Said et al. (33) put forward the hypothesis that a constrictor prostaglandin, $PGF_{2\alpha}$ is the mediator of HPV. This hypothesis was, however, refuted by investigators suggesting that endogenous vasodilator substances derived from arachidonic acid oppose HPV. This suggestion was based on the fact that indomethacine and meclofenamate potentiate HPV (34, 35). However, later attempts to isolate arachidonic acid metabolites from lung effluent blood or lung tissue have been unsuccessful (36). More recently, Garrett & Thomas (37) reported increased shunt fraction secondary to prostaglandin E_1 infusion in dogs with atelectasis as indirect evidence of abolished HPV. These researchers could not, however, find elevation of prostanoid metabolites in effluent blood from the atelectatic left lower lobe, and they found no correlation between shunt fraction and 6-keto-$PGF_{1\alpha}$ (38). Thus, most likely, a mediating role for these metabolites can be discarded unless they are so rapidly metabolized in tissue that they cannot be detected.

Among the leukotrienes, LTD_4 has proven to be a potent constrictor of the pulmonary vasculature. Moreover, it has been demonstrated that leukotriene inhibitors block HPV, but results from different species are rather ambiguous as regards specificity, thus making leukotrienes unlikely for the role as the only mediator (39–43).

Calcium

Constriction of vascular smooth muscle requires an increase in the cytosolic Ca^{2+} concentration. Suggestedly peptidoleukotrienes acting as calciumionophores (particularly LTD_4 increased Ca^{2+} inward current activity) enhance both receptor operated (ROC) and potential operated (POC) channels (43). This may stimulate cellular uptake and release of Ca^{2+} from stores in endoplasmatic reticulum.

Calcium channel blockers like verapamil and nifedipine dampen HPV (44, 45). Conversely, enhanced responses have been observed after administration to the perfusate of isolated rat lungs or to intact dogs of BAY K8644, a calcium channel potentiator (46). These findings are supported by the observation that strips of small pulmonary arteries (< 300 μm diam) constrict in response to hypoxia at concomitant depolarization of smooth muscle cell membrane (47). Harder (48) and Hottenstein et al. (49) have suggested that the electrical response to hypoxia of pulmonary artery smooth muscle cell membrane is largely the consequence of increased Ca^{2+} permeability; the membrane potential changes and action potentials generated depend predominantly on Ca^{2+} influx.

In recent years another class of agents has attracted attention: the phosphoinositides emanating from the phospholipid (50). There is abundant evidence that these agents, acting via Ca^{2+} mobilization, play an important role in signal transduction from receptors at the plasma membrane. The polyphosphoinositides have also been shown to contain a large share of arachidonates. It is, however, not known what proportion of total arachidonates liberated is derived from these agents and what the mechanism of release is.

Inositol triphosphate (IP_3) appears to be an important link between receptor-activated phosphoinositide breakdown and Ca^{2+} mobilization from intracellular stores such as the endoplasmatic reticulum. Breakdown of IP_3 is associated with Ca^{2+} re-uptake. It has been suggested that phosphoinositide metabolism plays a role in the opening of Ca^{2+} gates at the plasma membrane to permit entry of extracellular Ca^{2+}.

Diacylglycerol is a further cleavage product of phosphoinositides. The most important role of this metabolite seems to be activation of protein kinase C which is a cytosolic enzyme predominantly causing phosphorylation. Thus, stimuli causing breakdown of polyphosphoinositides result in formation of IP_3, diacylglycerol and protein kinase C. Suggestedly, these agents act in a synergistic manner to yield a maximum physiological response (50).

Inhalation anesthetics inhibit HPV in animal models (7). As far as human beings are concerned, results are ambiguous; when given in low to moderate concentrations, isoflurane and enflurane do not depress HPV (51, 52) whereas diethyl ether and halothane administered at high concentrations have a dampening effect (53). The mechanisms underlying these effects are unknown, but as far as halothane and isoflurane are concerned, calcium channel blocking effects have been suggested (54, 55). Thus, Ca^{2+} undoubtedly plays an important role in the chain of events initiating vascular smooth muscle contraction, although the criteria of being a specific mediator of HPV have not as yet been settled.

Toxic oxygen metabolites

Several investigators have suggested that TOM regulate vascular reactivity (56). If TOM are important for elicitation of HPV, it might be pertinent to ask whether substances like hydrogen peroxide, superoxide anions and hydroxyl anions are produced under conditions of tissue hypoxia. We believe that the enzyme system xanthine dehydrogenase/xanthine oxidase (XD/XO) may provide a key to the question. This system interferes both with intracellular purine metabolites and TOM. However, an obvious mechanism linking TOM to HPV does not exist. TOM are constantly produced in proportion to the tissue oxygen tension. Increased generation has been observed in lung tissue during exposure to toxic oxygen concentrations (57). XD/XO is sensitive to the intracellular oxygen tension. Tissue anoxia and ischemia suggestedly promote conversion of XD to XO. Consequently, increased TOM-levels may occur even though access to oxygen is diminished. In plasma perfused lungs it was observed that intravascularly administered XO induced pulmonary vasoconstriction of the same magnitude whether ventilation was with 2% O_2 or normoxic (58). Anyhow, one should bear in mind that arachidonic acid metabolites may result from the reaction between TOM and cellular membranes. Though it is an attractive idea, we have no clearcut explanation as to whether or not TOM are involved in the elicitation of HPV.

Endothelium derived relaxing and constricting factors

A few years ago investigators found that TOM modulate contractions of vascular smooth muscle. In experiments on canine coronary artery rings, hydrogen peroxide proved to inhibit acetylcholine released endothelium derived relaxing factor (EDRF). Superoxide anions depressed and hydroxyl radicals facilitated endothelium-dependent relaxations caused by activation of muscarinic receptors (59).

More recent investigations have provided evidence for hydrogen peroxide relaxation of pre-contracted bovine pulmonary artery rings being independent of EDRF or prostaglandins (PG's). Responses were markedly attenuated by methylene blue which inhibits the activation of soluble guanylate cyclase, the transformation enzyme of 3',5'-cyclic guanosine monophosphate (cGMP). Micromolar concentrations of hydrogen peroxide elicited increments in arterial cGMP that were associated with vascular relaxations. The increase was antagonized by the superoxide anion and by inactivation of catalase, a scavenger of the hydroxyl anion (56). A most interesting observation referred to above, i.e. that small muscular arteries contract when exposed to hypoxia, is supposed to be due to inactivation of $\cdot O^{2-}$ (the active agent of EDRF) by superoxide anions: $\cdot NO + \cdot O_2^- = ONOO^-$ (peroxonitrite anion) (60). However, the interaction of TOM and endothelium derived relaxing and constricting factors is not yet completely established and we still have a long way to go before we can explain the puzzling chain of events eliciting HPV.

VASCULAR CHANGES IN ARDS – IS HPV INVOLVED?

Arterial hypoxemia is the ultimate result of lung injury in ARDS. The microvascular hyperpermeability is the single most recognized pathogenetic factor of edema formation. Additionally, many cases are associated with pulmonary arterial hypertension due to enhancement of PVR, although left ventricular end-diastolic filling pressure will as a rule stay normal. In seriously ill patients, an up to four-fold increase in PVR may ensue, raising mean pulmonary arterial pressure (PAP) above 40 mmHg (61).

The acute lung edema starts at the corner vessels of the alveolus, probably due to the higher surface tension in this region, and extends to the alveoli (62). Loss of surfactant increases surface tension and enhances edema formation due to more subatmospheric pressures in the alveolar wall. A vicious circle may arise, resulting in small airway closure and microatelectasis. In lungs that have become atelectatic it is well docu-

mented that the rise in PVR is mainly caused by HPV (63, 64). Thus, unless blocked by ARDS mediators or therapeutic measures, we believe that HPV might contribute to the increase in PVR. At a late stage of edema, microvessels may become leaky both to plasma constituents and blood cells and, at the worst, the result will be generalized lung fibrosis (61, 62, 65, 66). As far as this condition is concerned, reports on HPV are circumstantial. In a study of two cases of cystic fibrosis indirect evidence has been obtained suggesting that HPV is present even at an advanced stage of disease (66).

When evaluating ARDS pathophysiological changes, anatomical alterations of the pulmonary artery lumen should be taken into account. In an angiographic study, Greene et al. recognized that 80 out of 220 patients had multiple widespread pulmonary artery filling defects. At autopsy most victims had pulmonary thromboembolism (67). Confirmatory evidence has been obtained by other investigators, who additionally observed marked thickening of the muscularis media and growth of smooth muscle into distal parts of the pulmonary arterial bed in longstanding ARDS (68, 69). Most likely, the resulting scatter in blood flow distribution will cause redistribution towards non-obstructed microvessels. The latter will be exposed to higher hydrostatic pressures which add to the increased permeability in contracting pulmonary edema. Moreover the increment in pulmonary pressure will blunt HPV thereby contributing to increased PVR and impaired arterial oxygen tension (70, 71).

ARDS mediators and HPV

A survey of suggested mediators of ARDS is presented in Fig. 2. Several mechanisms proposedly act in concert to bring about the changes in PVR, some of which are constrictors, others dilators. Activation of complement causing activation and aggregation of polymorphonuclear leucocytes (PMN) seems to initiate the process (72). The subsequent release of TOM, lysosomal enzymes and arachidonic acid metabolites damage the endothelium (73). In the most severe cases of septicemia-induced ARDS even monocyte-released thromboplastin may be at play triggering disseminated intravascular coagulation (DIC) (74).

Out of the variety of arachidonic acid derivatives, thromboxane A_2 (TXA_2), a product of the cycloxygenase pathway, is a potent vasoconstrictor. TXA_2 is released into the pulmonary circulation of rabbits both *in vivo* and in isolated lungs perfused with Krebs-Henseleit-albumin solution free of blood cells indicating that TXA_2 may be released directly from pulmonary tissue (74). It seems to be generally accepted that the profound pulmonary vasoconstriction in early ARDS is mediated by intrapulmonary released and/or produced TXA_2. Other vasoconstrictor PG's such as $PGF_{2\alpha}$, PGE_2 and PGD_2 may add to the picture (75). We have no direct information about the effect of these PG's on HPV but investigations on dog left lower lobe preparations and isolated rat lungs have revealed that HPV is hampered at high pulmonary artery transmural pressures (70, 71).

Prostacyclin (PGI_2) is the most potent vasodilator in the lung circulation. A few years ago, investigators suggested that endotoxin inhibition of HPV is a result of selective increase in endogenous production of prostacyclin. They presented two types of evidence in support of this theory: first, in the late phase of endotoxemia HPV was absent, but could be restored by treatment with the cycloxygenase inhibitor meclofenamate; second, infusion of arachidonic acid during HPV resulted in vasodilation coincident with increased lung production of prostacyclin metabolites (76). To a certain extent, other PG's may also contribute to vascular dilation out of which PGE_1 is by far the most investigated HPV inhibitor (76, 77).

Demonstration of increased concentrations of leucotrienes in lung edema and lavage fluid from experimental animals and patients with ARDS supports the assumption that lipoxygenase products may also be at play (42). The leucotrienes possess the capability of altering vascular reactivity and permeability. Furthermore, they have been shown to be potent stimulators of neutrophil chemotaxis and adhesion to the vascular endothelium. As far as effects on HPV are concerned, the various metabolites differ. Whereas LTD_4 is a constrictor of pulmonary vessels (43) and has been suggested to take part in mediation of HPV, other intermediates dilate pulmonary vessels.

Platelet aggregation is an important incidence in the development of ARDS causing release of serotonin (5-hydroxytryptamine; 5-HT), which stimulates both bronchial and vascular smooth muscle. 5-HT induces vasoconstriction of pulmonary arterioles and veins resulting in pulmonary hypertension (78). Sibbald et al. observed that patients with sepsis-induced ARDS had a fall in platelet concentration, but a significantly higher plasma 5-HT concentration as compared to controls (79). Further evidence of 5-HT release has been obtained by Demling et al. who noticed that ketanserin, a selective $5\text{-}HT_2$ receptor blocker, abolished pulmonary hypertension occurring at a late phase of endotoxin-induced ARDS in sheep (80). In a recent study on rat isolated lungs, ketanserin administered at a concentration abolishing pressor responses to high doses of 5-HT had no effect on hypoxia-induced increments in PVR (81). These two

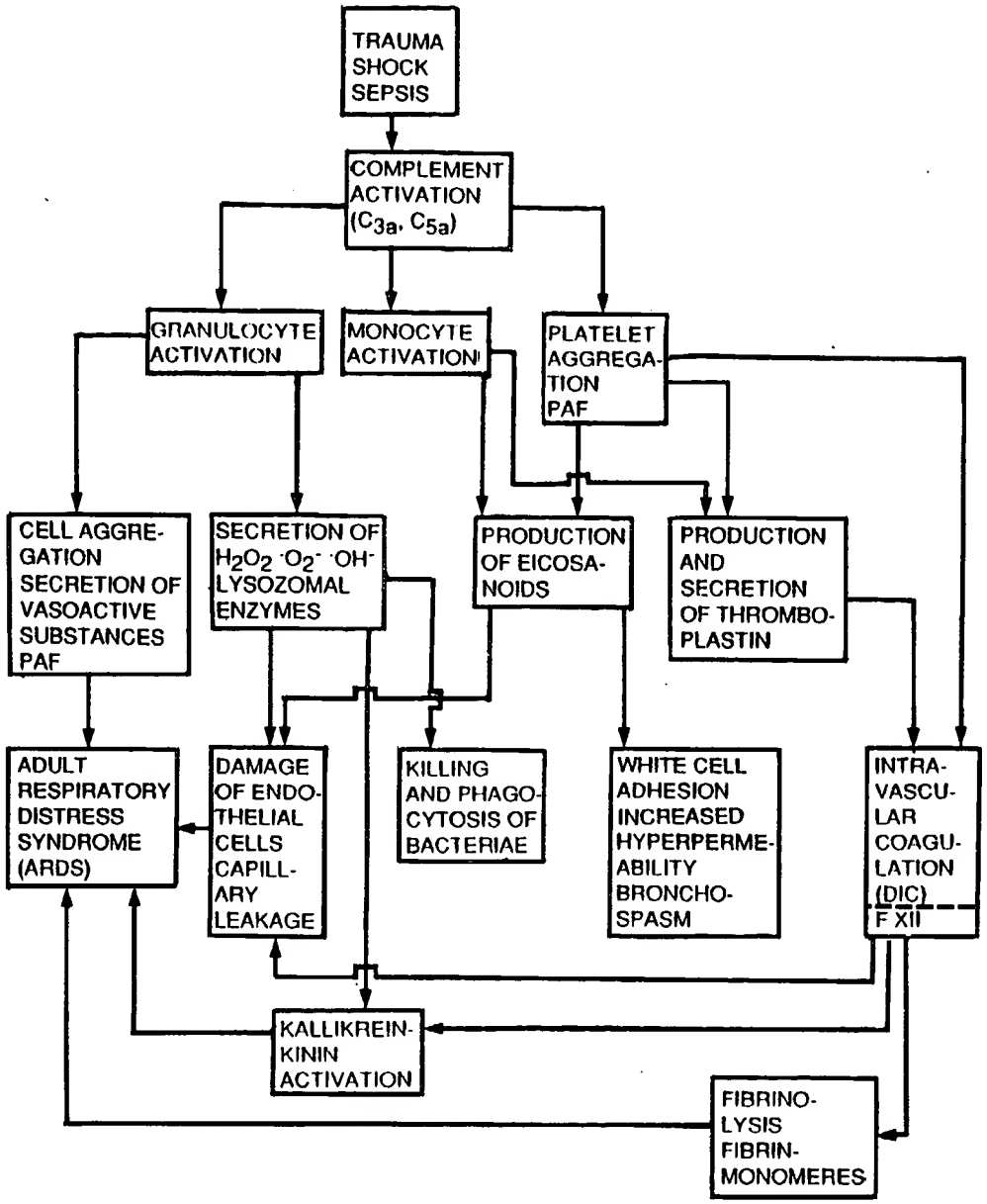


Fig. 2. Cellular and humoral systems suggested to be activated in patients with ARDS. See text for details.

studies suggest that platelet sequestration and 5-HT release are involved in the rise in PVR in endotoxin-induced ARDS, even though serotonin *per se* apparently does not affect HPV.

According to Ashbaugh et al. (82) the coagulation system is always activated in shock and other conditions that may often be complicated by ARDS. In the most severe cases, activation may be extensive including both cell- and cascade systems as in fullblown DIC. A literature review provided no exact information about the effects on HPV of coagulation factors, but according to Neuhof et al. (74) fibrinopeptides and fibrin monomers, cleavage products of fibrinogen by thrombin-induced proteolysis, are prone to increase both pulmonary vascular tone and permeability.

Also the kinin-kallikrein system is activated in ARDS patients (83). Activation is concomitant with coagulation, fibrinolytic and complement systems (84). The resulting production of bradykinin increases permeability and so brings about a worsening of the syndrome which, most likely, includes derangement of

HPV. An exciting hypothesis forwarded by De Oliveira & de Oliveira Antonio (85) suggests that the pathophysiology associated with ARDS might be a consequence of cybernetic derangement of the stress-adapting mechanisms of the central nervous system, probably due to development of "alarm-reaction-induced" cerebrovascular microthrombosis (86, 87) damaging hypothalamic centers. Expression of this damage is by activation of several peripheral effectors, some being ultimately responsible for ARDS development. Among the activated endogenous substances, the kinins and the catecholamines act together to cause the hyperpermeability, and inhibition of either of the substances will hamper the development of ARDS (85).

In an investigation employing New Zealand white rabbits, Nuytinck and co-workers (65) recently noticed that the combination of zymosan-activated complement and short hypoxic episodes apparently aggravates the lung microvascular injury with occurrence of protein-rich alveolar edema and hemorrhage. Since complement-activation is an important step in the evolution of ARDS, it seemed reasonable to examine whether activated complement affects HPV in its own right. In dogs, HPV was reduced by infusion of zymosan-activated complement. This effect was partly blocked by meclofenamate suggesting that inhibition involves dilator prostaglandin-like substances (88).

Platelet activating factor (PAF) (a phospholipid) is released from inflammatory cells known to be sequestered in the lung during evolution of injury (89). This mediator, which is supposed to be released during ARDS, has recently been shown to depress HPV and vasoconstriction in response to angiotensin II (90). Thus, an abundance of mediators released by the disease process acts synergistically to bring about the vascular changes in ARDS, some of them, most likely, interfering with HPV.

THERAPEUTIC INTERVENTIONS AFFECTING HPV

Strategies to improve survival from ARDS require artificial ventilation with positive end-expiratory pressure (PEEP) and, in the most severe cases, extracorporeal lung assistance device. Adjuvant therapy includes agents that modulate PVR, antagonize hyperpermeability and enhance right ventricular contractility. Here we will focus on the first group of substances. As a matter of fact, HPV may be influenced not only by its own mediators and those ensuing from the evolution of ARDS, but also from superimposed interventions. Facing the individual ARDS patient we should consider whether HPV is an advantageous mechanism to be preserved at the cost of increased PVR or an unfavorable mechanism which should be antagonized. The decision whether HPV should be preserved or not, or even strengthened, depends on which intervention gives the best outcome in terms of oxygen delivery.

Effects of artificial ventilation with PEEP

There exists no exact information about the effect on HPV of continuous positive pressure ventilation. In lungs subjected to acute respiratory failure it has been possible to differentiate between normally inflated, poorly inflated and non-inflated regions (91). Impairment of gas exchange was directly correlated to non-inflated lung tissue mass as a fraction of total lung weight. The quantity of normally inflated areas correlated positively with the PaO_2 and negatively with the shunt fraction. This might imply that HPV, most likely, is intact in normally inflated areas. Extrapolating this information to patients with ARDS, we hypothesize that during artificial ventilation employing PEEP, the most "normal" respiratory units will receive a greater part of the tidal volume due to lower airway resistance and higher compliance as compared to the less aerated units (91). As a result of relative hyperinflation, zone I conditions arising in the former units may divert blood towards more poorly aerated units. Since, however, the net effect of a continuous airway pressure is improvement of arterial oxygen tension, the above effect might be outweighed by the advantageous increase in gas exchange area and redistribution of extravascular lung water secondary to PEEP (62).

Vasoactive agents suggested to interfere with HPV

In clinical settings it is difficult to interpret whether a dilating agent acts by purely abolishing pulmonary vasoconstriction, by recruiting the vascular bed or both. Table 1 surveys studies of ARDS pin-pointing effects of vasoactive drugs suspected of interference with HPV. The Table includes both potentiators and inhibitors of HPV. If arterial hypoxemia exists in the presence of low PVR, therapy should aim at restoring HPV. Recent studies in animals have documented enhancement of HPV by low dose almitrine bismesylate (92). This finding has been confirmed in healthy humans (93) as well as in patients with ARDS (94) with improvement of arterial oxygenation. Thus, although information is scanty, we have reasons to believe that potentiators of HPV, like almitrine bismesylate, circumstantially may improve oxygenation.

In victims of ARDS high PVR may impede right ventricular function and reduce cardiac output ($\dot{Q}$). Hence, a vasodilating agent may prove useful because it enhances $\dot{Q}$ and, possibly also, mixed venous oxy-

Table 1

Clinical and experimental studies of ARDS showing pulmonary hemodynamic- and gas exchange changes suspected of being influenced by hypoxic pulmonary vasoconstriction (HPV). Indications of HPV have been derived from hemodynamic- and gas exchange alterations caused by inhibitors and potentiators of HPV. None of these studies aimed at specifically investigating HPV. It is therefore impossible to distinguish between blunting of HPV and vascular recruitment, and information in the table should therefore be considered as hypothetical.
PVR = pulmonary vascular resistance, CO = cardiac output, PaO_2 = arterial oxygen tension, Q_s/Q_t intrapulmonary right-to-left shunt.

Ref. No.	Species	Agents	HPV	PVR	CO	PaO_2	Q_s/Q_t
95	Human	SNP	↓	↔	↔	↓	↑
		KET	↓	↔	↔	↓	↓
99	Human	PGE_1	↔	↓	↑	↑	↑
103	Human	DILT	↓	↓	↔	↓	↑
97	Human	PGE_1	↓	↓	↑	↓	↑
		NTG	↓	↓	↑	↓	↑
100	Human	PGI_2	↓	↓	↑	↔	↑
104	Dog	MIN	↓	↓	↔	↔	↑
98	Dog	NTG	↓	↓	↓	↓	↑
96	Dog	SNP	↓	↓	↔	↓	↑
94	Human	ALM	↑	↑	↔	↑	↓
106	Human	MP	↓	↓	↑	↓	↑
109	Rat isolated lungs	ADEN	↓	↓	↔	↔	↔

↑ = increase
↓ = decrease
↔ = unchanged

ADEN = adenosine
ALM = almitrine bismesylate
DILT = diltiazem
KET = ketamine hydrochloride
MIN = minoxidil
MP = methylprednisolone
NTG = nitroglycerine
PGE_1 = prostaglandin E_1
PGI_2 = prostacyclin
SNP = sodium nitroprusside

gen tension (PvO_2). On the other hand, vasodilation *per se* may preclude a drop in arterial oxygen tension (PaO_2) due to increased intrapulmonary shunt causing saturation to fall along the oxyhemoglobin dissociation curve. This effect may jeopardize oxygen delivery calculated as the product of arterial oxygen content and CO.

Recently, investigators have reported from studies on patients with mild to moderate ARDS that sodium nitroprusside infusion caused a fall in PAP and a rise in venous admixture. As pulmonary capillary wedge pressure and CO remained essentially the same, it is tempting to hypothesize that the reduction of PVR, at least in part, was secondary to attenuation of HPV (95). This investigation supports an earlier report documenting impairment of HPV during infusion of sodium nitroprusside in dogs subjected to oleic acid-induced pulmonary edema (96). Thus, increased distribution of pulmonary circulation to poorly oxygenated regions appears to be the most likely explanation of the fall in PaO_2 upon infusion of this agent (61, 95).

Some years ago, investigators noted that PGE_1 inhibits HPV (76). In patients with mild to moderate ARDS Radermacher et al. (97) compared the effects on pulmonary hemodynamics and gas exchange of nitroglycerin and PGE_1 at a dosage rate aiming at a 20% reduction in mean arterial pressure. At the dosages tested both drugs reduced PVR. Nitroglycerin induced a fall in PaO_2 and a rise in venous admixture caused by increased distribution of perfusion to non-inflated areas and regions of low ventilation-perfusion ratios. Conversely, pulmonary capillary wedge pressure, CO and PvO_2 remained unaltered. The authors concluded that the changes were probably due to inhibition of HPV, thereby confirming previously reported blunting of HPV during nitroglycerin infusion in can-

ine oleic acid-induced lung injury (97, 98). As far as PGE_1 is concerned, findings were principally the same, except for an increment in oxygen delivery which was mainly attributable to augmentation of CO. Enhancement of CO also partly explains the decrease in PVR secondary to PGE_1 infusion (97). In a similar group of patients other investigators have obtained corresponding results but for the difference that PvO_2 remained unchanged during infusion of PGE_1 (99).

In a further study, Radermacher et al. (100) recently evaluated the vasodilating properties of prostacyclin. They found a fall in PAP and PVR and an increase in venous admixture due to significant deterioration of ventilation-perfusion distributions. The fall in right ventricular afterload resulted for the major part from the vasodilating effect of prostacyclin causing CO and PvO_2 to increase by 35% and 4.9 mmHg, respectively. The increase in shunt did not affect PaO_2 thereby leaving arterial oxygen content nearly unchanged. Both prostacyclin and high PvO_2 reportedly impair HPV, but the study design does not allow for any conclusion as regards the quantitative contribution of each of the inhibitors to the total reduction of HPV (101, 102). Thus, in spite of a most likely impairment of HPV, prostacyclin improves CO and oxygen delivery in patients with ARDS.

A recent investigation of patients with moderate ARDS revealed that diltiazem, a calcium channel blocker, induces pulmonary vasodilation with a reduction in PAP and PVR without any significant change in CO. The effect was accompanied by deterioration of pulmonary gas exchange as evidenced by the multiple inert gas elimination technique. The dominant finding was a diversion of blood flow from normally ventilated and perfused compartments to shunt compartments. Whether this effect was due to vasodilatation or recruitment was not possible to decide, but most likely, it could be explained in terms of inhibition of HPV (103). The latter observation was supported by the demonstration of reduced HPV secondary to infusion of minoxidil in dogs subjected to oleic acid-induced lung injury (104). Also other investigators have reported dampening of HPV following administration of calcium channel blockers (45).

Previous experiments on rat isolated lungs have demonstrated that methylprednisolone (MP) reduces HPV. This finding has also been confirmed in man (105, 106). A few years ago, a study of patients with lung contusion subsequent to blunt chest trauma showed that MP injected at a dose of 30 mg/kg body weight reduced PVR at the cost of increased intrapulmonary shunt. Cardiac index also increased albeit not in proportion to the fall in PVR, while PvO_2 concomitantly increased (107). We interpret these changes as being partly caused by a dampening effect on HPV. MP inhibits phospholipase A_2 which may start the derangement of arachidonic acid. MP also hampers complement activation and aggregation of granulocytes (108). In pulmonary hypertension due to ARDS, PVR was reduced by MP. This reduction might well partly be an effect of loss of HPV (106).

Adenosine is a potent vasodilator of both the systemic and the pulmonary circulation with a half-life in blood of less than ten seconds. When administered at low dosage via the tip of a pulmonary artery catheter, adenosine seems advantageous because it restricts its action more or less to the pulmonary circulation. In cases of life-threatening increment of right ventricular impedance it is particularly important to reduce PVR with the least possible influence on diastolic systemic blood pressure which is a major determinant for myocardial perfusion. In isolated rat lungs adenosine has been shown to reduce HPV in a dose-dependent fashion (109). The relatively low concentrations necessary for obtaining this effect seem promising from the point of view of limiting the effect on the lesser circulation. Adenosine additionally has the capability of preventing pulmonary vascular leakage in fatty acid-induced lung injury (110). As yet, neither of these effects has been tested in clinical settings of ARDS and further investigations are warranted.

In animal experiments TOM-induced lung damage could be prevented by the simultaneous administration of superoxide dismutase and catalase, implying that production of superoxide by granulocytes is important in the mediation of cell injury (111). An increased amount of extracellular TOM may also occur during the course of the hypoxanthine-xanthine oxidase reaction. To counteract this oxidative stress, antioxidant defenses exist, whether enzymatic, like superoxide dismutase and catalase, or nonenzymatic, like vitamin E (112). In cases of endotoxin-induced ARDS, the use of human monoclonal antibodies against tumor necrosis factor has also shown encouraging results and may become an integrated part of future therapy of ARDS (113).

In conclusion, the present review supports the likelihood that HPV contributes to the increased PVR at an early state of disease in ARDS. Moreover, results from experimental studies suggest that mediators released during ARDS may interact with HPV and hamper a possible beneficial effect of this mechanism on arterial oxygenation. Also, drugs used in the treatment of ARDS may abolish HPV at the cost of reduced arterial oxygenation. Although being an inhibitor of HPV, prostacycline appears to be the most advantageous vasodilating agent because oxygen delivery is enhanced due to increased CO. However, adenosine

with a half-life of only a few seconds seems promising and may become an integrated part of ARDS therapy in the future; however, further investigations are warranted.

ACKNOWLEDGEMENTS

We thank Carmeda AB, Stockholm, Sweden for generously supporting us with an electronic data telecommunication system.

REFERENCES

1. Bachhofen M, Weibel E R. Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin Chest Med* 1982: 3: 35–56.
2. Euler U S von, Liljestrand G. Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol Scand* 1946: 12: 301–320.
3. Gray W, Gray M D. High altitude pulmonary edema. *Sem Respir Med* 1983: 5: 141–149.
4. Grover R F, Vogel J H, Averill K H, Blunt S G. Pulmonary hypertension. Individual and species variability relative to vascular reactivity. *Am Heart J* 1963: 66: 1–3.
5. Fishman A P. Hypoxia on the pulmonary circulation. How and where it acts. *Circ Res* 1976: 38: 221–231.
6. Gottlieb J E, Peake M D, Sylvester J T. Adenosine and pulmonary vasodilation. *Am J Physiol* 1984: 247: H541–H547.
7. Bjertnaes L J. Hypoxia-induced vasoconstriction in isolated perfused rat lungs exposed to injectable and inhalation-anaesthetics. *Acta Anaesthesiol Scand* 1977: 21: 133–147.
8. Hauge A, Melmon K L. Role of histamine in hypoxic pulmonary hypertension in the rat. Depletion of histamine, serotonin and cathecolamines. *Circ Res* 1968: 12: 385–392.
9. Hauge A, Staub N C. Prevention of hypoxic pulmonary vasoconstriction in cat lung by histamine-releasing agent 48/80. *J Appl Physiol* 1969: 26: 693–699.
10. Aviado D M, Samanek M, Folle L E. Cardiopulmonary effects of tobacco and related substances. *Arch Environ Health* 1966: 12: 705–712.
11. Haas F, Bergofsky E H. Role of the mast cell in the pulmonary pressor response to hypoxia. *J Clin Invest* 1972: 51: 3154–3162.
12. Logaras G. Further studies of the pulmonary arterial blood pressure. *Acta Physiol Scand* 1947: 14: 120–126.
13. Barer G R. Reactivity of the vessels of collapsed and ventilated lungs to drugs and hypoxia. *Circ Res* 1966: 18: 366–378.
14. Barer G R, Howard P, McCurrie J R, Shaw J W. Changes in the pulmonary circulation after bronchial occlusion in the anesthetized dogs and cats. *Circ Res* 1969: 25: 747–764.
15. Barer G R, McCurrie J R. Pulmonary vasomotor responses in the cat: the effect and interrelationships of drugs, hypoxia and hypercapnia. *Q J Exp Physiol* 1969: 54: 156–172.
16. Thilenius O G, Candiolo B M, Beug J I. Effect of adrenergic blockade on hypoxia induced pulmonary vasoconstriction in awake dogs. *Am J Physiol* 1967: 213: 990–998.
17. Hauge A. Role of histamine in hypoxic pulmonary hypertension in the rat. I. Blockade or potentiated of endogenous amines, kinins, and ATP. *Circ Res* 1968: 22: 371–383.
18. Malik A B, Kidd B S L. Adrenergic blockade and the pulmonary vascular response to hypoxia. *Respir Physiol* 1973: 19: 96–106.
19. Cassin S, Dawes G S, Ross B B. Pulmonary blood flow and vascular resistance in immature foetal lambs. *J Physiol* (Lond) 1964: 171: 80–89.
20. Howard P, Barer G R, Thompson B, Warren P M, Abbott C J, Mungall I P F. Factors causing and reversing vasoconstriction in unventilated lung. *Respir Physiol* 1975: 24: 325–345.
21. Mentzer R M Jr, Alegre C A, Nolan S. The effects of dopamine and isoproterenol on the pulmonary circulation. *Thorac Cardiovasc Surg* 1976: 71: 807–814.
22. Marin J L B, Orchard C, Chakrabarti M K, Sykes M K. Depression of hypoxic pulmonary vasoconstriction in the dog by dopamine and isoprenaline. *Brit J Anaesth* 1979: 51: 303–312.
23. Fordham R M M, Resnekov L. Arterial hypoxemia. A side-effect of intravenous isoprenaline used after cardiac surgery. *Thorax* 1968: 23: 19–23.
24. Knudson R J, Constantine H P. An effect of isoproterenol on ventilation-perfusion in asthmatic versus normal subjects. *J Appl Physiol* 1967: 22: 402–406.
25. Chick T W, Nicholson D P, Johnson R L Jr. Effects of isoproterenol on distribution of ventilation and perfusion in asthma. *Am Rev Respir Dis* 1973: 107: 869–873.
26. Levitzki A. Catecholamine receptors. *Rev Physiol Biochem Pharmacol* 1978: 82: 1–26.
27. Sill V, Kaukel E, Völkel N, Siemssen S. The significance of cyclic 3′-5′-AMP for the Euler-Liljestrand mechanism. *Pneumonologie* 1974: 150: 337–344.
28. Rhoades R A, Whittle E G. Selective action of hypoxia on rat cyclic AMP. *Resp Physiol* 1978: 35: 59–63.
29. Völkel N, Duschak W, Kaukel E, Beier W, Siemssen S, Sill V. Histamine – an important mediator for the Euler-Liljestrand mechanism? *Pneumonologie* 1975: 152: 113–121.
30. Bisgaard G E, Will J A. Glucagon and aminophylline as pulmonary vasodilators in the calf with hypoxic pulmonary hypertension. *Chest* 1977: 71 (Suppl. 2): 263–265.
31. Porcelli R J, Viau A, Demeny M, Naftchi N E, Bergofsky E H. Relation between hypoxic pulmonary vasoconstriction, its humoral mediators and alpha-beta adrenergic receptors. *Chest* 1977: 71 (Suppl. 2): 249–251.
32. Hammond B A, Kontos H A, Hess M L. Oxygen radicals in the adult respiratory distress syndrom, in myocardial ischemia and reperfusion injury and in cerebral vascular damage. *Can J Physiol Pharmacol* 1985: 63: 173–187.
33. Said S I, Yoshida T, Kitamura S, Wreim C. Pulmonary alveolar hypoxia: Release of prostaglandins and other humoral mediators. *Science* 1974: 185: 1181–1183.
34. Vaage J, Bjertnæs L, Hauge A. The pulmonary vasoconstrictor response to hypoxia: Effects of inhibitors of prostaglandin biosynthesis. *Acta Physiol Scand* 1975: 95: 95–101.
35. Weir E K, McMurtry I F, Tucker A, Reeves J T, Grover R F. Prostaglandin synthetase inhibitors do not decrease hypoxic pulmonary vasoconstriction. *J Appl Physiol* 1976: 41: 714–718.
36. Wiberg T, Vaage J, Bjertnæs L, Hauge A, Gautvik K M. Prostaglandin content in blood and lung tissue during alveolar hypoxia. *Acta Physiol Scand* 1978: 102: 181–190.
37. Thomas H M, Garrett R C. Strength of hypoxic vasoconstriction determines shunt fraction in dogs with atelectasis. *J Appl Physiol* 1982: 53 (1): 44–51.
38. Garrett R C, Thomas H M. Relation of prostanoids to strength of hypoxic vasoconstriction in dogs with lobar atelectasis. *J Appl Physiol* 1985: 59: 72–77.
39. Ahmed T, Oliver W. Does slow-reacting substance of anaphylaxis mediate hypoxic pulmonary vasoconstriction? *Am Rev Respir Dis* 1983: 127: 566–571.
40. Morganroth M L, Reeves J T, Murphy R C. Leukotriene synthesis and receptor blockers block hypoxic pulmonary vasoconstriction. *J Appl Physiol* 1984: 56: 1340–1345.
41. McCormack D G, Paterson N A M. The contrasting influence of two lipoxygenase inhibitors on hypoxic pulmonary vasocon-

striction in anesthetized pigs. *Am Rev Respir Dis* 1989: **139**: 100–105.
42. Sprague R S, Stephenson A H, Dahms T E, Lonigro A J. Proposed role for leukotrienes in the pathophysiology of multiple systems organ failure. *Crit Care Clin* 1989: 5: 315–329.
43. Skryma R N, Kalashnikova L E, Degtiar W E, Prevarskaia N B, Burdiga F V. Action of leukotriene LTC_4 on the calcium channels of the somatic membrane of the nerve cell and tone of smooth muscle organs. *Neirofiziologiia* 1989: **21**: 262–264.
44. McMurtry J F, Davidson A B, Reeves J T, Grover R F. Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. *Circ Res* 1976: **38**: 99–104.
45. Steinbrook H S, Morris K G, McMurtry I F. Prevention and reversal of hypoxic pulmonary hypertension by calcium antagonists. *Am Rev Respir Dis* 1984: **130**: 81–85.
46. Tolins M, Weir E K, Chesler E, Nelson D P, From A H. Pulmonary vascular tone is increased by a voltage-dependent calcium channel potentiator. *J Appl Physiol* 1986: **60**: 942–948.
47. Madden J A, Dawson C A, Harder D R. Hypoxia-induced activation in small isolated pulmonary arteries from the cat. *J Appl Physiol* 1985: **59**: 113–118.
48. Harder D R. Pressure-dependent membrane depolarization in cat middle cerebral artery. *Circ Res* 1984: **55**: 197–202.
49. Hottenstein O D, Mitzner W A, Bierkamper G G. Hypoxia alters membrane potentials in rat main pulmonary arteries (abstract). *Federation Proc* 1984: **43**: 923.
50. Sekar M C, Hokin L E. The role of phosphoinositides in signal transduction. *J Membrane Biol* 1986: **89**: 193–210.
51. Carlsson A J, Bindslev L, Hedenstierna G. Hypoxia-induced pulmonary vasoconstriction in the human lung. The effect of isoflurane anesthesia. *Anesthesiology* 1987: **66**: 312–316.
52. Carlsson A J, Hedenstierna G, Bindslev L. Hypoxia-induced vasoconstriction in human lung exposed to enflurane anaesthesia. *Acta Anaesthesiol Scand* 1987: **31**: 57–62.
53. Bjertnæs L J. Hypoxia-induced pulmonary vasoconstriction in man: Inhibition due to diethylether and halothane anaesthesia. *Acta Anaesthesiol Scand* 1978: **22**: 570–588.
54. Chelly J E, Rogers K, Hysing E S, Taylor A, Hartley C, Merin R G. Cardiovascular effects of and interactions between calcium blocking drugs and anesthetics in chronically instrumented dogs. I. Verapamil and halothane. *Anesthesiology* 1986: **64**: 560–567.
55. Rogers K, Hysing E S, Merin R G, Taylor A, Hartley C, Chelly J E. Cardiovascular effects of and interaction between calcium blocking drugs and anesthetics in chronically instrumented dogs. II. Verapamil, enflurane and isoflurane. *Anesthesiology* 1986: **64**: 568–575.
56. Burke T M, Wolin M S. Hydrogen peroxide elicits pulmonary arterial relaxation and guanylate cyclase activation. *Am J Physiol* 1987: **252**: H721–H732.
57. Freeman B A, Crapo J D. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* 1981: **256**: 10986–10992.
58. Kjæve J, Vaage J, Bjertnæs L. Factors governing the vascular and airway reponses induced by toxic oxygen metabolites in isolated perfused rat lungs. Role of proteases, complement and arachidonic acid metabolites. In manuscript.
59. Rubanyi G M, Vanhoutte P M. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am J Physiol* 1986: **250**: H822–H827.
60. Beckman J S, Beckman T W, Freeman B A. Endothelial cell-derived relaxing factor and vascular free radical metabolism. *Am Rev Respir Dis* 1989: **139**: A55.
61. Zapol W M, Snider M T, Rie M, Frikker M, Quinn. Pulmonary circulation during ARDS. In: W M Zapol and K Falke eds. Acute respiratory failure, in the series Lung Biology in Health and Disease. New York: Marcel Dekker, 1985: 241–273.
62. Staub N C. State of the art review. Pathogenesis of pulmonary edema. *Am Rev Respir Dis* 1974: **109**: 358–372.
63. Marshall B E. Importance of hypoxic pulmonary vasoconstriction with atelectasis. *Adv Shock Res* 1982: **8**: 1–12.
64. Bjertnæs L, Mundal R, Hauge A, Nicolaysen A. Vascular resistance in atelectatic lungs: effects of inhalation anaesthetics. *Acta Anaesthesiol Scand* 1980: **24**: 109–118.
65. Nuytinck J K, Goris R J, Weerts J G, Schillings P H, Stekhoven J H. Acute generalized microvascular injury by activated complement and hypoxia: the basis of the adult respiratory distress syndrom and multiple organ failure? *Br J Exp Pathol* 1986: **67**: 537–548.
66. Michael J R, Kennedy T P, Fitzpatrick S, Rosenstein B J. Nifedipine inhibits hypoxic pulmonary vasoconstriction during rest and exercise in patients with cystic fibrosis and cor pulmonale. *Am Rev Respir Dis* 1984: **130**: 516–519.
67. Greene R, Boggis C R M, Jantsch H S. Radiography and angiography of the pulmonary circulation in ARDS. In: W M Zapol and K Falke eds. Acute respiratory failure, in the series Lung Biology in Health and Disease. New York: Marcel Dekker, 1985: 275–302.
68. Jones R, Reid L M, Zapol W M, Tomashefski J F, Kirton O C, Kobayashi K. Pulmonary vascular pathology: human and experimental studies. In: W M Zapol and K Falke eds. Acute Respiratory failure, in the series Lung Biology in Health and Disease. New York: Marcel Dekker, 1985: 23–160.
69. Tomashefski J F, Zapol W M, Reid L M. The pulmonary vascular lesions of the adult respiratory distress syndrome. *Am J Pathol* 1983: **112**: 112–126.
70. Bjertnæs L, Hauge A. Constant flow vs. constant pressure-perfusion for studies of pulmonary vascular responses. *Acta Physiol Scand* 1980: **109**: 193–200.
71. Benumof J L, Wahrenbrock E A. Blunted hypoxic pulmonary vasoconstriction by increased lung vascular pressures. *J Appl Physiol* 1975: **38**: 846–850.
72. Jacob H S. The role of activated complement and granulocytes in shock states and myocardial infarction. *J Lab Clin Med* 1981: **29**: 645–690.
73. Neuhof H, Seeger W, Wolf H R D. Generation of mediators by limited proteolysis during blood coagulation and fibrinolysis – its pathogenetic role in the adult respiratory distress syndrome (ARDS). *Resuscitation* 1986: **14**: 23–32.
74. Bjertnæs L. Plasma exchange in septic shock. In: G Schlag and H Redl, eds. Progress in clinical and biological research, vol. 236 B. First Vienna shock forum. N.Y.: Allan Liss, 1987: 215–223.
75. Brigham K, Bowers R, McKeen C. Methylprednisolone prevention of increased lung vascular permeability following endotoxemia in sheep. *J Clin Invest* 1981: **67**: 1103–1110.
76. Weir E K, Reeves J T, Grover R F. Prostaglandin E_1 inhibited the pulmonary vascular pressor response to hypoxia and prostaglandin F_2 alpha. *Prostaglandins* 1975: **10**: 623–631.
77. Sprague R S, Stephenson A H, Heitmann L J, Lonigro A J. Differential response of the pulmonary circulation to prostaglandins E_2 and $F_{2\alpha}$ in the presence of unilateral alveolar hypoxia. *J Pharm Exp Therapy* 1984: **229**: 38–43.
78. Huval W V, Lelcuk S, Shepro D, Hechtman H B. Role of serotonin in patients with acute respiratory failure. *Ann Surg* 1984: **200**: 165–172.
79. Sibbald W J, Peters S, Lindsay R M. Serotonin and pulmonary hypertension in human septic ARDS. *Crit Care Med* 1980: **8**: 490–494.
80. Demling R H, Wong C, Fox O et al. Relationship of increased lung serotonin levels to endotoxin-induced pulmonary hyper-

tension in sheep: Effect of a serotonin antagonist. *Amer Rev Respir Dis* 1985: **132**: 1257–1261.
81. Helgesen K G, Bjertnæs L. The effect of ketanserin on hypoxia-induced vasoconstriction in isolated lungs. *Int J Microcirc: Clin Exp* 1986: **5**: 65–72.
82. Ashbaugh D G. Respiratory distress syndrome. Respiratory diseases. National Institute of Health. DHEW Publication 1972: No 73-432: 165–180.
83. Herrera C, Velasco F, Guerrero A, Guerrero F, Alvarez F, Torres A. Contact phase of blood coagulation in cardiogenic pulmonary oedema (CPO) and adult respiratory distress syndrome. *Intensive Care Med* 1989: **15**: 99–104.
84. Margolis J. Activation of plasma by contact with glass: Evidence for a common reaction which releases plasma kinin and initiates coagulation. *J Physiol* (Lond) 1958: **144**: 1–22.
85. De Oliveira G G, de Oliveira Antonio M P. Adult respiratory distress syndrome (ARDS): The pathophysiologic role of catecholamine-kinin interactions. *J Trauma* 1988: **28**: 246–253.
86. De Oliveira G G, Shimano L T, de Oliveira Antonio M P. Acute respiratory distress syndrome (ARDS): The prophylactic effect of neurodepressant agents. *J Trauma* 1986: **26**: 451–457.
87. Moss G, Staunton C, Stein A A. Cerebral etiology of "shock lung syndrome". *J Trauma* 1972: **12**: 885–890.
88. Weir K, Tierney J F, Chesler E, Lundquist L J, Craddock P R. Zymosan activation of plasma reduces hypoxic pulmonary vasoconstriction. *Respir Physiol* 1983: **53**: 295–306.
89. Rinaldo J E, Rogers R M. Adult respiratory distress syndrome. Changing concepts of lung injury and repair. *New Engl J Med* 1982: **306**: 900–909.
90. Gillespie M N, Bowdy B D. Impact of platelet activating factor on vascular responsiveness in isolated rat lungs. *J Pharmacol Exp Therapy* 1986: **236**: 396–402.
91. Gattinoni L, Pesenti A, Bombino M, Baglioni S, Rivolta M, Rossi F, Rossi G, Fumagalli R, Marcolin R, Mascheroni D, Torresin A. Relationships between lung computed tomographic density, gas exchange, and PEEP in acute respiratory failure. *Anesthesiology* 1988: **69**: 824–832.
92. Chen I., Miller F L, Clarke W R, Clergue F, Marshall C, Marshall B E. Low-dose almitrine bismesylate enhances hypoxic pulmonary vasoconstriction during canine one-lung hypoxia. *Anesthesiology* 1987: **67**: A548.
93. Mèlot C, Dechamps P, Hallemans R, Decroly P, Mols P. Enhancement of hypoxic pulmonary vasoconstriction by low dose almitrine bismesylate in normal humans. *Am Rev Respir Dis* 1989: **139**: 111–119.
94. Reyes A, Roca J, Roderiguez-Roisin R, Torres A, Ussetti P, Wagner P. Effect of almitrine on ventilation-perfusion distribution in adult respiratory distress syndrome. *Am Rev Respir Dis* 1988: **137**: 1962–1067.
95. Radermacher P, Huet Y, Pluskwa F, Herigault R, Mal H, Teisseire B, Lemaire F. Comparison of Ketanserin and sodium-nitroprusside in patients with severe ARDS. *Anesthesiology* 1988: **68**: 152–157.
96. Colley P S, Cheney F W, Hlastala M P. Ventilation-perfusion and gas exchange effects of sodium nitroprusside in dogs with normal and edematous lungs. *Anesthesiology* 1979: **50**: 489–495.
97. Radermacher P, Santak B, Becker H, Falke K J. Prostaglandin E_1 and nitroglycerin reduce pulmonary capillary pressure but worsen ventilation-perfusion distributions in patients with adult respiratory distress syndrome. *Anesthesiology* 1989: **70**: 601–606.
98. Colley P S, Cheney F W, Hlastala M P. Pulmonary gas exchange effects of nitroglycerin in canine edematous lungs. *Anesthesiology* 1981: **55**: 114–119.
99. Melot C, Lejeune P, Leeman M, Moraine J J, Naeije R. Prostaglandin E_1 in the adult respiratory distress syndrome. *Am Rev Respir Dis* 1989: **139**: 106–110.
100. Radermacher P, Santak B, Wust H J, Tarnow J, Falke K J. Prostacyclin for the treatment of pulmonary hypertension in the adult respiratory distress syndrom: Effects on pulmonary capillary pressure and ventilation-perfusion distributions. *Anesthesiology* 1990: **72**: 238–244.
101. Sprague R S, Stephenson A H, Lonigro A J. Prostaglandin I_2 supports blood flow to hypoxic alveoli in anesthetized dogs. *J Appl Physiol* 1984: **56**: 1246–1251.
102. Domino K B, Wetstein L, Glasser S A, Lindgren L, Marshall C, Harken A, Marshall B E. Influence of mixed venous oxygen tension (PvO_2) on blood flow to atelectatic lung. *Anesthesiology* 1983: **59**: 428–434.
103. Melot C, Naeije R, Mols P, Hallemans R, Lejeune P, Jaspar N. Pulmonary vascular tone improves pulmonary gas exchange in the adult respiratory distress syndrome. *Am Rev Respir Dis* 1987: **136**: 1232–1236.
104. Bishop M J, Huang T, Cheney F W. Effect of vasodilator treatment on the resolution of oleic acid injury in dogs. *Am Rev Respir Dis* 1985: **131**: 421–425.
105. Strömme-Hansen L, Bjertnæs L J, Vaage J. Methylprednisolone reduces vascular resistance in hypoxic and atelectatic lungs. *Acta Anaesthesiol Scand* 1985: **29**: 446–452.
106. Vaage J, Peterson C, Bjertnæs L. Methylprednisolone inhibits hypoxic pulmonary vasoconstriction in man (abstract). *Int J Microcirc: Clin Exp* 1984: **3**: 78.
107. Svennevig J L, Bugge-Asperheim B, Vaage J, Geiran O, Birkeland S. Corticosteroids in the treatment of blunt injury of the chest. *Injury* 1984: **16**: 80–84.
108. Brigham K, Bowers R, McKeen C. Methylprednisolone prevention of increased lung vascular permeability following endotoxemia in sheep. *J Clin Invest* 1981: **67**: 1103–1110.
109. Jolin Å, Kjæve J, Hambraeus K, Sollevi A, Bindslev L, Bjertnæs L J. The influence of adenosine on hypoxia-induced pulmonary vasoconstriction in isolated perfused rat lungs (abstract). *J Cardiothor Anesth* 1989: **3**: 33.
110. Helset E, Jolin Å, Kjæve J, Dahl P E, Bjertnæs L. The effect of exogenously administered adenosine on pulmonary hemodynamics and fluid filtration rate after lung injury (abstract). *Int J Microcirc: Clin Exp* 1990: In press.
111. Zimmerman J J, Shelhamer J H, Parrillo J E. Quantitative analysis of polymorphonuclear leukocyte superoxide anion generation in critically ill children. *Crit Care Med* 1985: **13**: 143–150.
112. Junod A F. Oxygen free radicals and lungs. *Intensive Care Med* 1989: **15**: 21–23.
113. Beutler B, Cerami A. Cachectine: more than a tumor necrosis factor. *New Engl J Med* 1987: **316**: 379–385.

Address:
Åse Jolin, M.D.
Department of Anesthesiology
Institute of Clinical Medicine
University of Tromsø
N-9000 Tromsø
Norway

DISCUSSION

Participants: Dag Lundberg, Heinz Neuhof, Lars Irestedt, Göran Hedenstierna, Keith Sykes, Lars Bindslev

Lundberg: Is it so that the hypoxic vasoconstrictive ability is found only in the vessels of the pulmonary circuit?

Bjertnæs: Yes, as far as I know this is so. But of course if you strip the arteries then you can see this constriction in other kinds of arteries; if you remove for example the endothelial layer – I think this was shown by Lloyd as early as the 1960's.

Lundberg: There must be some specific thing in that very area then?

Bjertnæs: Yes, and especially Jane Maiden's recent experiments I think show that it is precisely the very small arteries that constrict when exposed to hypoxia, not the aorta and not the main pulmonary artery. It seems to be confined to just these very small arteries.

Lundberg: What about different species? Is it general or what?

Bjertnæs: It seems to be general, but there are differences between the species and I don't think that the rabbit is the best responder.

Neuhof: I think you can agree that there is a great discrepancy in the literature concerning the mediators. We have made a lot of experiments on isolated lungs – they are not yet published. But it seems as if there are two different modes we are speaking of. This maybe clears the discrepancies. One that hypoxic pulmonary vasoconstriction is a physiological mechanism which regulates the blood flow between ventilated and non-ventilated areas. Superimposed is a mechanism by an unphysiological stimulation of arachidonic acid metabolism which can injure the lung by barotrauma, for instance, and cause a pulmonary oedema. There are differences reported in the literature between animals. In the dogs, there is a diminishing of the response if you block the cyclooxygenase pathways. In the rabbit it is quite different, the thromboxanes are the main mediators, etc. I think the most interesting question – and it has not yet been answered – is how the human being responds. Do we respond like rabbits, is thromboxane the main mediator, or do we respond like ferrets or what?

Bertnæs: The question is very interesting. In opposition to Brian Marshall I don't think that this mechanism is so very important in adult life. I think it has its greatest role in the fetal circulation. And therefore I also think that the observation we made that adenosine is not generated at length during hypoxia is in opposition to what Menser found many years ago, because he did not isolate the pulmonary circulation, so I think the adenosine he got came from other organs. Anyhow it would have been advantageous if adenosine had been liberated because then under hypoxic conditions in the fetal lung, the pulmonary circulation would have opened up and the lungs would have been more blood flooded and the placenta less. As of the species differences I think they are more difficult to comment on because I really don't know the reason for them.

Irestedt: Adenosine might be involved in the patency of the ductus, for instance, and when the adenosine level falls after delivery this might be one of the reasons why the ductus closes.

Bjertnæs: But what is very interesting concerning this substance is that it tightens the pulmonary vasculature during an injury. I know that it has been shown for isoprenaline, terbutaline and aminophylline – agents that increase the cyclic-AMP – that these are substances with a very short halflife; and maybe we could use adenosine more selectively than the other drugs, even than prostacyclin despite the fact that prostacyclin has been shown to be the most promising agent up to now. I think we should definitively examine also this function of adenosine.

Hedenstierna: I must say that what you briefly touched upon was a new proposal of how attenuation of hypoxic pulmonary vasoconstriction may be brought about. We are then no longer looking for a substance that is the mediator of HPV but rather one the reduced production of which is the cause, for instance NO. Is there more support for what you stated rather briefly that reduced production of NO may be the cause of HPV?

Bjertnæs: I think the answer lies in the oxygen free radicals. It may be that oxygen free radical can be produced even under hypoxic conditions by turning from xantine dehydrogenase to xantine oxydase. Maybe it is a balance because we see that the oxygen free radicals obviously play an important role in the regulation of the microcirculation, and so I

think we have to examine also these kinds of substances.

Sykes: I would rather like to be critical of the idea that there is active HPV in ARDS because I think in the vast majority of those studies you quote you could find some other factor which was altering the distribution, and I really don't think there is any strong evidence for this. If you could find me one paper that could convince me I would be happy.

Bjertnæs: I completely agree, it is no strong evidence and we called it indirect evidence. We have a suspicion, and the first EMCO patient in Tromsö demonstrated a picture which could be explained by just eliciting HPV because at the same time as his PaO_2 went up his pulmonary arterial pressure also went up; and what happened to his cardiac output – I unfortunately only have the measurements after treatment.

Sykes: I don't think you have convinced me!

Bjertnæs: I think you have to take this as a more casuistic demonstration.

Bindslev: Do you think the HPV reflex is rudimentary in mammalian animals and actually out of business in humans; where the reflex should be used is in diving animals – there it is really advantageous; but we are studying human beings, or other mammalians, and looking for something which is disappearing.

Bjertnæs: It is an interesting question although I think it is somewhat wrong because we are talking about two different responses: the hypoxic response is one, and the diving response is another; but you are right, I think too they are both rudimentary responses though of course in dramatic lifethreatening situations they are activated; it is of course not life-threatening to have atelectasis, but it is a situation that threatens the oxygen uptake, at least.

1: Thromb Res 1996 Feb 1;81(3):315-326 Related Articles, Books,

TNF-alpha suppresses IL-6 upregulation of protein S in HepG-hepatoma cells.

Hooper WC, Phillips DJ, Evatt BL.

Hematologic Diseases Branch, Centers for Disease Control and Prevention, A Ga 30333, USA.

The pathogenesis of disseminated intravascular coagulation (DIC) has, in part attributed to the impairment of the natural anticoagulant protein C/protein S p DIC, which frequently occurs during sepsis, has been linked to cytokines that induce or modulate procoagulant activity. Three of these cytokines, IL-1 alph and TNF-alpha have been reported to be increased in the early stages of sepsi present study, we have stimulated HepG-2 hepatoma cell cultures with recom human IL-1 alpha, IL-6, TNF-alpha, and oncostatin M (OSM). The results demonstrated that TNF-alpha, and to a lesser degree, IL-1 alpha, could signifi suppress IL-6 upregulation of protein S, whereas the effects of OSM was onl suppressed by the combination of IL-1 alpha and TNF-alpha. The combinatio IL-1 alpha and TNF-alpha also suppressed protein S production below that of or basal levels. These results indicate that IL-1 alpha and TNF-alpha may play important regulatory roles in coagulation.

PMID: 8928089 [PubMed - indexed for MEDLINE]

1: Tumori 1996 Jan;82(1):78-80 Related Articles, Books,

Characterization of cancer-related disseminated intravascular coagulation in relation to tumor necrosis factor-alpha blood concentrations: possible therapeutic role of pentoxifylline.

Lissoni P, Ardizzoia A, Barni S, Pittalis S, Rossini F, Porta A, Tancini G

Division of Radiation Oncology, San Gerardo Hospital, Monza (Mi), Italy.

AIMS AND BACKGROUND: Preliminary experimental data suggest the involvement of tumor necrosis factor (TNF) in determining endothelial damag related to disseminated intravascular coagulation (DIC). The present study wa performed to investigate TNF secretion in DIC occurring in metastatic solid t patients and to evaluate the possible therapeutic role of pentoxifylline, which h proven to have a TNF-lowering activity. METHODS: The study included 20 metastatic solid tumor patients who showed clinical and laboratory signs of D Pentoxifylline was given orally at a dose of 1200 mg/day for 28 days. RESUL Abnormally high levels of TNF were found in 13/20 patients, and mean TNF levels observed in patients were significantly higher than those seen in a contr of 50 healthy subjects. Fibrinogen plasma concentrations were low in 11 case Patients with low fibrinogen values showed significantly higher mean TNF lev those with normal or elevated concentrations. Pentoxifylline therapy induced a significant decrease in mean TNF concentrations and a significant increase in platelet number, which returned to within the normal range in 11/20 patients. increase in platelets in response to pentoxifylline was more evident in patients elevated pretreatment TNF values. CONCLUSIONS: Our results suggest the existence of abnormally high blood levels of TNF in cancer-related DIC, main presence of low fibrinogen values. Moreover, they indicate that pentoxifylline may determine a decrease in TNF levels in DIC patients, an event associated increase in platelet number.

PMID: 8623512 [PubMed - indexed for MEDLINE]

Display Abstract Sort Save Text Add to Clipboard

1: Cytokine 1995 Jan;7(1):15-25 Related Articles, Books,

The mouse/human chimeric monoclonal antibody cA2 neutraliz TNF in vitro and protects transgenic mice from cachexia and T lethality in vivo.

Siegel SA, Shealy DJ, Nakada MT, Le J, Woulfe DS, Probert L, Kollias (Ghrayeb J, Vilcek J, Daddona PE.

Department of Immunology, Centocor, Inc., Malvern PA 19355, USA.

The pleiotropic cytokine tumour necrosis factor-alpha (TNF) is thought to pla central role in infectious, inflammatory and autoimmune diseases. Critical to th understanding and management of TNF-associated pathology is the developm highly specific agents capable of modifying TNF activity. We evaluated the at high affinity mouse/human chimeric anti-TNF monoclonal antibody (cA2) to neutralize the in vitro and in vivo biological effects of TNF. cA2 inhibited TNF-induced mitogenesis and IL-6 secretion by human fibroblasts, TNF-prim human neutrophils, and the stimulation of human umbilical vein endothelial ce TNF as measured by the expression of E-selectin, ICAM-1 and procoagulant cA2 also specifically blocked TNF-induced adherence of human neutrophils t(endothelial cell monolayer. Receptor binding studies suggested that neutraliza resulted from cA2 blocking of TNF binding to both p55 and p75 TNF recepto the cells. In vivo, repeated administration of cA2 to transgenic mice that constitutively express human TNF reversed the cachectic phenotype and preve subsequent mortality. These results demonstrated that cA2 effectively neutrali broad range of TNF biological activities both in vitro and in vivo.

PMID: 7538333 [PubMed - indexed for MEDLINE]

1: Thromb Haemost 1993 Feb 1;69(2):164-172 Related Articles, Books,

Inflammatory and procoagulant mediator interactions in an experimental baboon model of venous thrombosis.

Wakefield TW, Greenfield LJ, Rolfe MW, DeLucia A 3rd, Strieter RM, GD, Kunkel SL, Esmon CT, Wrobleski SK, Kadell AM, et al.

Jobst Research Laboratories, Department of Surgery, University of Michigan Center, Ann Arbor 48109-0329.

Theoretic and in vitro evidence suggests that thrombosis and inflammation are interrelated. The purpose of the present study was to define the relationship be inflammation and deep venous thrombosis (DVT) in an in vivo model. Initiati DVT was accomplished by administration of antibody to protein C (HPC4, 2 and tumor necrosis factor (TNF, 150 micrograms/kg); stasis; and subtle veno catheter injury. Thrombosis was assessed by thrombin-antithrombin assay (TA 125I-fibrinogen scanning (scan) over both the proximal and distal iliac veins, a ascending venography. Cytokines TNF, interleukin-6 (IL-6), monocyte chemc protein-1 (MCP-1), and interleukin-8 (IL-8) were measured along with differe white blood cell counts, platelet counts, fibrinogen (FIB), and erythrocyte sedimentation rates (ESR). Baboon pairs were sacrificed on day 3 (T + 3d), T and T + 9d and veins removed. All animals developed inferior vena cava and l iliofemoral DVT by venography; no right DVT was found. TAT was elevated 1hr and peaked at T + 3hrs. Left iliofemoral DVT was found at T + 1hr by sca reached a 20% uptake difference between the affected left and nonaffected rig at T + 3hrs. TNF peaked at T + 1hr; MCP-1 peaked at T + 6hrs; IL-8 and IL- peaked on T + 2d; all cytokines declined to baseline. TNF and TAT elevation: found to correlate with all cytokines; elevations in IL-8 were correlated with elevations in MCP-1 and IL-6 ($p < 0.05$).(ABSTRACT TRUNCATED AT 2: WORDS)

PMID: 8456429 [PubMed - indexed for MEDLINE]

1: Lab Invest 1988 Apr;58(4):365-378 Related Articles, Books,

Acute inflammation and microthrombosis induced by endotoxi interleukin-1, and tumor necrosis factor and their implication i gram-negative infection.

Cybulsky MI, Chan MK, Movat HZ.

Department of Pathology, University of Toronto, Ontario, Canada.

Publication Types:
- Review
- Review, tutorial

PMID: 3282123 [PubMed - indexed for MEDLINE]

WEST

Help | Logout | Interrupt

Main Menu | Search Form | Posting Counts | Show S Numbers | Edit S Numbers | Preferences

Search Results -

Terms	Documents
l31 and @ad<19960216	3

Database: US Patents Full-Text Database
US Pre-Grant Publication Full-Text Database
JPO Abstracts Database
EPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Refine Search: `l31 and @ad<19960216` Clear

Search History

Today's Date: 9/16/2001

DB Name	Query	Hit Count	Set Name
USPT	l31 and @ad<19960216	3	L32
USPT	l13 with ((coagul$5) or (anti-coagul$5))	5	L31
USPT	l13 same ((coagul$5) or (anti-coagul$5))	25	L30
USPT	l13 and ((coagul$5) or (anti-coagul$5))	229	L29
JPAB,EPAB,DWPI	l26 and coagulat$3	10	L28
JPAB,EPAB,DWPI	l26 (coronar$3 or thromb$ or infarction$1 or stroke$1 or (heart adj disease$1))	0	L27
JPAB,EPAB,DWPI	l18 or l20 or l25	252	L26
JPAB,EPAB,DWPI	antibod$3 near3 l24	98	L25
JPAB,EPAB,DWPI	l21 or l22 or l23	2631	L24
JPAB,EPAB,DWPI	(tumor or tumour) adj necrosis adj (factor-alpha)	140	L23
JPAB,EPAB,DWPI	(tumor or tumour) adj necrosis adj (factor-a)	4	L22
JPAB,EPAB,DWPI	(tumor or tumour) adj necrosis adj factor	2631	L21

JPAB,EPAB,DWPI	antibod$3 near3 l19	180	L20
JPAB,EPAB,DWPI	(TNF) or (TNFa) or (aTNF) or (TNF-a) or (a-TNF) or (TNFalpha) or (TNF-alpha) or (alphaTNF) or (alpha-TNF)	3117	L19
JPAB,EPAB,DWPI	(anti-TNF) or (anti-TNFa) or (anti-aTNF) or (anti-TNF-a) or (anti-a-TNF) or (anti-TNFalpha) or (anti-TNF-alpha) or (anti-alphaTNF) or (anti-alpha-TNF)	100	L18
USPT	l16 and @ad<19960216	14	L17
USPT	l13 same (coronar$3 or thromb$ or infarction$1 or stroke$1 or (heart adj disease$1))	28	L16
USPT	l14 and @ad<19960216	153	L15
USPT	l13 and (coronar$3 or thromb$ or infarction$1 or stroke$1 or (heart adj disease$1))	342	L14
USPT	l5 or l11 or l12	760	L13
USPT	antibod$3 near3 l9	251	L12
USPT	anti adj l9	84	L11
USPT	anti-l9	0	L10
USPT	l6 or l7 or l8	5653	L9
USPT	(tumor or tumour) adj necrosis adj (factor-alpha)	1249	L8
USPT	(tumor or tumour) adj necrosis adj (factor-a)	51	L7
USPT	(tumor or tumour) adj necrosis adj factor	5653	L6
USPT	l2 or l4	632	L5
USPT	antibod$3 near3 l3	563	L4
USPT	(TNF) or (TNFa) or (aTNF) or (TNF-a) or (a-TNF) or (TNFalpha) or (TNF-alpha) or (alphaTNF) or (alpha-TNF)	5705	L3
USPT	(anti-TNF) or (anti-TNFa) or (anti-aTNF) or (anti-TNF-a) or (anti-a-TNF) or (anti-TNFalpha) or (anti-TNF-alpha) or (anti-alphaTNF) or (anti-alpha-TNF)	356	L2
USPT	TNF or TNFa or (TNF-a) or (TNF-alpha) or (a-TNF) or (alpha-TNF) or ((tumor or tumour) adj necrosis adj factor)	7733	L1

```
=> S TNF OR ATNF OR TNFA OR TNFALPHA OR ((TUMOR OR
TUMOUR)(W)NECROsis(w)factor)
         14098 TNF
            44 TNFS
         14103 TNF
                 (TNF OR TNFS)
             0 ATNF
            82 TNFA
             4 TNFALPHA
         60024 TUMOR
         26943 TUMORS
         70947 TUMOR
                 (TUMOR OR TUMORS)
         10212 TUMOUR
          7616 TUMOURS
         14976 TUMOUR
                 (TUMOUR OR TUMOURS)
         23188 NECROSIS
        137745 FACTOR
        125971 FACTORS
        229547 FACTOR
                 (FACTOR OR FACTORS)
         14914 (TUMOR OR TUMOUR)(W)NECROSIS(W)FACTOR
L8       18045 TNF OR ATNF OR TNFA OR TNFALPHA OR ((TUMOR OR
TUMOUR)(W)NECROSIS
               (W)FACTOR)

=> s anti(w)l8
         77100 ANTI
L9         708 ANTI(W)L8

=> s l8(3a)antibod?
        154212 ANTIBOD?
L10        836 L8(3A)ANTIBOD?

=> s l9 or l10
L11       1122 L9 OR L10

=> s l11(s)(INFARCTION OR CORONARY OR THROMBO? OR STROKE OR
(HEART(W)DISEASE#))
          1810 INFARCTION
           144 INFARCTIONS
          1877 INFARCTION
                 (INFARCTION OR INFARCTIONS)
          2972 CORONARY
            10 CORONARIES
          2974 CORONARY
                 (CORONARY OR CORONARIES)
          7536 THROMBO?
          2012 STROKE
           220 STROKES
          2134 STROKE
                 (STROKE OR STROKES)
         27421 HEART
          2343 HEARTS
         27948 HEART
                 (HEART OR HEARTS)
```

```
        181834 DISEASE#
L12          13 L11(S)(INFARCTION OR CORONARY OR THROMBO? OR STROKE OR
(HEART(W)
                DISEASE#))

=> s l12 and py<1997
       1645819 PY<1997
L13          11 L12 AND PY<1997

=> d ibib abs tot
```

```
     FILE 'MEDLINE, BIOSIS, LIFESCI' ENTERED AT 17:22:00 ON 16 SEP 2001
L1       134325 S ((TUMOR OR TUMOUR)(W)NECROSIS(W)FACTOR) OR TNF OR TNFA OR
ATN
L2         4634 S ANTI(W)L1
L3         5951 S ANTIBOD?(3A)L1
L4         7572 S L2 OR L3
L5           63 S L4(S)COAGUL?
L6           35 DUP REM L5 (28 DUPLICATES REMOVED)
L7           26 S L6 AND PY<1997
L8          689 S L1(3A)ANTAGONIST#
L9           35 S L8 AND (INFARCT? OR CORONAR? OR STROKE# OR THROMBO? OR COAGU
L10          13 S L9 AND PY<1997
L11           7 DUP REM L10 (6 DUPLICATES REMOVED)

=> log h
```